Chronic mild social stress increases neurogenesis in adolescent male rats

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Abstract

Once thought to occur only during specific periods of development, it is now clear that neurogenesis occurs in the rat hippocampus into adulthood. It is well-established that stress during adulthood decreases the rate of neurogenesis, but during adolescence, the effects of stress are much less understood. I investigated the effect of short-term or chronic stress during adolescence (daily 1hr isolation and change of cage partner from postnatal day (PND) 30-32 or 30-45) on hippocampal neurogenesis. In experiment 1, rats were administered Bromodeoxyuridine (BrdU) daily on PND 30-32, or 46-48, to mark neurogenesis at the beginning of the stressor or after the stressor had ceased, respectively. Neither short-term nor chronic stress had an effect on proliferation or survival (evidenced by BrdU and Doublecortin (Dcx) immunohistochemistry respectively) of cells born at the beginning of the stress procedure. Compared to controls, BrdU-labeling showed chronic stress significantly increased proliferation of cells generated after the stressor had ceased, but survival of new neurons was not supported (Dcx-labeling). However, it may be that BrdU injections are inherently stressful. In experiment 2, the stressor (described above) was applied in the absence of BrdU injections. Ki67 (a marker of proliferation) showed that stress transiently increased cell proliferation. Dcx-labeling showed that stress also increased neuron survival into adulthood. Labeling with OX-42 (a marker of macrophages) suggested that the immune system plays a role in neurogenesis, as stress transiently decreased the number of activated microglia in the hippocampus. It can be concluded that in the adolescent male rat, chronic mild stress increases neurogenesis.

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Abbreviations

^{3}H-thymidine	Tritiated Thymidine
5-HT	Serotonin (5-Hydroxytryptamine)
ACTH	Adrenocorticotropic Hormone
ACUC	Animal Care and Use Committee
AVP	Arginine Vasopressin
BBB	Blood-Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
BrdU	Bromodeoxyuridine
CCAC	Canadian Council on Animal Care
CNS	Central Nervous System
CORT	Corticosterone
CR3	Complement Receptor 3
CRH	Corticotrophin-Releasing Hormone
CSS	Chronic Social Stress
CTL	Control
DAB	Diaminobenzidine
Dcx	Doublecortin
DG	Dentate Gyrus
GCL	Granule Cell Layer
GFAP	Glial fibrillary acidic protein
GR	Glucocorticoid Receptor
HPA Axis	Hypothalamic-Pituitary-Adrenal Axis
i.p.	Intraperitoneal
ir	Immunoreactive
ITI	Inter-Trial Interval

LPS	Lipopolysaccharide
LV	Lateral Ventricles
ML	Molecular Layer
MR	Mineralocorticoid Receptor
NeuN	Nuclear Neuronal Marker
PNCA	Proliferating Cell Nuclear Antigen
PND	Postnatal Day
PTSD	Post-Traumatic Stress Disorder
PVN	Paraventricular Nucleus
SGZ	Subgranular Zone
SS	Social Stress
SubSS	Subacute Social Stress
SVZ	Subventricular Zone
VIP	Very Intense Purple

Introduction

Once thought to be restricted to specific periods of embryonic development (Altman, 1962), neurogenesis is now known to occur in the postnatal brain into adulthood. Evidence for postnatal neurogenesis has been found in many vertebrates, including birds (Barnea & Nottebohm, 1994), reptiles (Font et al., 2001), and mammals. (Altman, 1962). In the mammalian brain, neurogenesis occurs in the subgranular zone (SGZ) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles throughout adulthood and into senescence. There are also reports of adult neurogenesis in various other brain regions of mammals, albeit to a lesser degree (Altman, 1962; Bedard et al., 2002; 2006; Gould et al., 1999a; 2001; Bernier et al., 2002; Ahmed et al., 2008; Zhao et al., 2003). Nevertheless, only neurogenesis in the hippocampus and lateral ventricles has been reliably demonstrated time and time again.

Hippocampal neurogenesis, the focus of this thesis, is thought to be involved in certain types of memory formation, spatial learning, and mood regulation (see reviews by Becker & Wojtowicz, 2006; Balu & Lucki, 2009; Zhao et al., 2008; Abrous et al., 2005). It is, thus, relevant that postnatal neurogenesis is regulated by various environmental, physiological and chemical influences. Modulators can positively or negatively affect the proliferation, differentiation, and survival of neurons. Stress is a powerful regulator of neurogenesis, and it is proposed that stress exerts its effects on neurogenesis though the actions of glucocorticoid hormones, the primary glucocorticoid being corticosterone (CORT) in the rat, and cortisol in humans. The mechanisms by which glucocorticoids influence neurogenesis are not fully understood. There are many studies on the effects

of stress on neurogenesis, but most have involved adult, male rats. Thus, research in the effects of stress on the period of adolescence is lacking. I attempted to elucidate how chronic social stress affects cell proliferation and survival of neurons during adolescence in the rat – a time when the brain is still maturing, and is characterized by much higher rates of hippocampal neurogenesis (McDonald & Wojtowicz, 2005) compared to during adulthood.

Hippocampal Neurogenesis

The discovery that radioactively labeled ([^{3}H]) thymidine is exclusively incorporated into the DNA of dividing cells (Taylor et al., 1957) paved the way for the discovery that neurogenesis occurs in the adult brain (Altman, 1962; Altman & Das, 1967). Largely because [^{3}H]-thymidine autoradiography is time-consuming and requires a radioactive substrate, in more recent years, 5-Bromo-2'-deoxyuridine (BrdU), a synthetic analogue of thymidine (Gratzner, 1975; Gratzner, 1982), and/or endogenous labels that can be detected using immunohistochemistry have become the preferred methods for studying neurogenesis.

It is now accepted that neurogenesis within the hippocampus is generated in the SGZ, a small region lining the granule cell layer (GCL) of the dentate gyrus (DG; see Figures 1 and 2). Two types of neural progenitors – known as type 1 and type 2 cells - exist in SGZ. These progenitor cells have qualities reminiscent of astrocytes, leading some to hypothesize that hippocampal neurogenesis has glial origins (Seri et al., 2001; Filippov et al., 2003). The two subpopulations are morphologically distinct (Filippov et al., 2003), have different electrophysiological properties (Kempermann et al., 2004), and

express different molecular markers. In neuron development, it is thought that type 1 cells – rich in astrocytic marker glial fibrillary acidic protein (GFAP) and progenitor cell marker nestin – mature into type 2 cells, which are still positive for nestin, but no longer GFAP(Seri et al., 2001). These transient progenitor cells eventually mature into neurons.

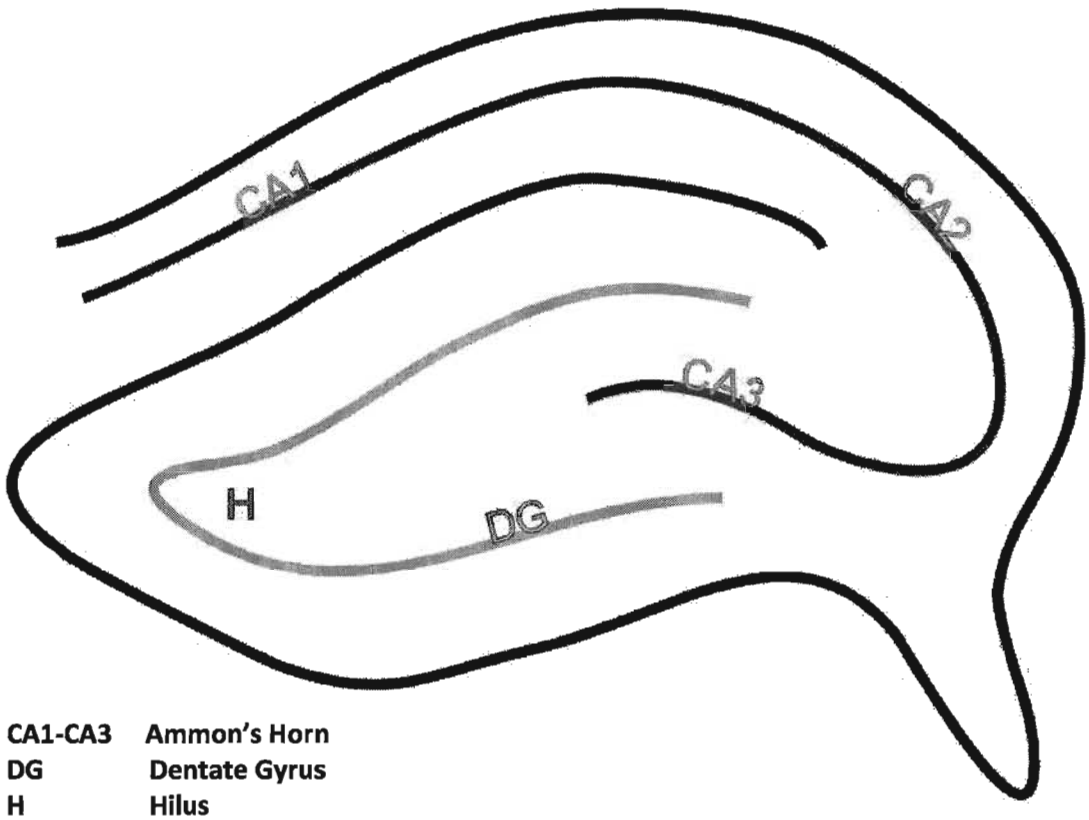

Figure 1. Schematic diagram of the hippocampus. The hippocampus, one located in each hemisphere. The CA1, CA2 and CA3 regions compose Ammon's horn, and the V-shaped portion is the dentate gyrus (DG). Also shown is the hilus (H).

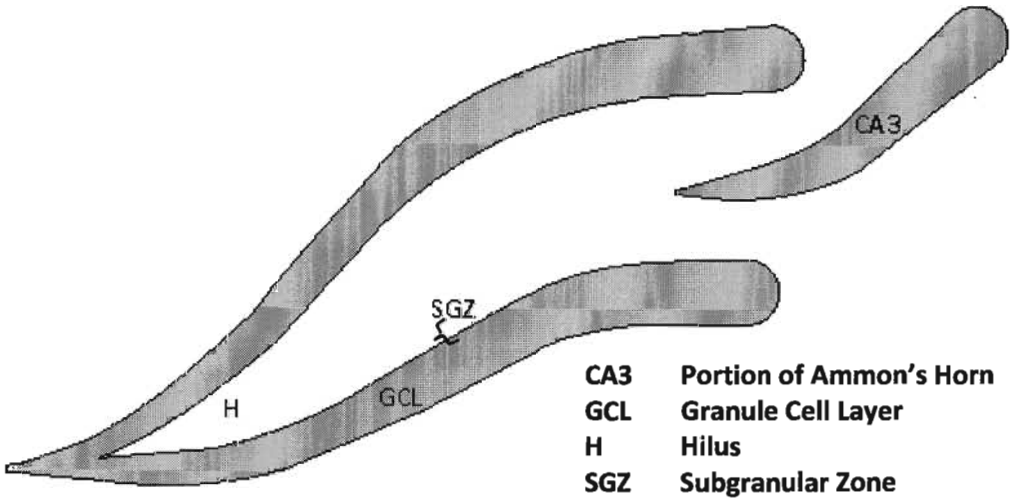

Figure 2. Schematic diagram of the dentate gyrus. The DG is further divided into the subgranular zone (SGZ) and granule cell layer (GCL), with the Hilus located in between the infrapyramidal and suprapyramidal blades of the DG – named as such because of their location relative to the CA3 region, also known as the pyramidal layer, which juts into the DG.

Neuron development from progenitor cell to mature neuron takes roughly eight weeks to complete, although different aspects of the neuron mature faster than others (reviewed in Zhao et al., 2008). No longer positive for molecular markers of stem cells, proliferative cells become positive for mitotic markers such as Ki67, and proliferating cell nuclear antigen (PCNA; Scholzen & Gerdes, 2000; see Figure 5). These cells migrate a short distance to the GCL, and gradually project dendritic trees into the molecular layer (ML), and axons into the hilus, and CA3 of region of Ammon's horn (Hastings & Gould, 1999). Within days, proliferative cells become classified as immature neurons and begin expressing doublecortin (Dcx), polysialylated neural adhesion molecule (PSA-NCAM; Brown et al., 2003), and other markers associated with neuron outgrowth. After roughly three weeks, neurons stop expressing immature neuron markers coincident with their expression of mature neuronal markers, such as nuclear neuronal marker

(NeuN; Brown et al., 2003). Four-week-old neurons resemble mature neurons, in that they project elaborate dendritic trees, axonal connections, and dendritic spines by this time (Zhao et al., 2006). However further maturation continues until about eight weeks of age, in the form of further dendritic arborization, spine development (Zhao et al., 2006), and synapse formation (Toni et al., 2007). Upon reaching maturation, neurons become integrated into the existing circuitry and become indistinguishable from the existing GCL population (van Praag et al., 2002; Toni et al., 2008).

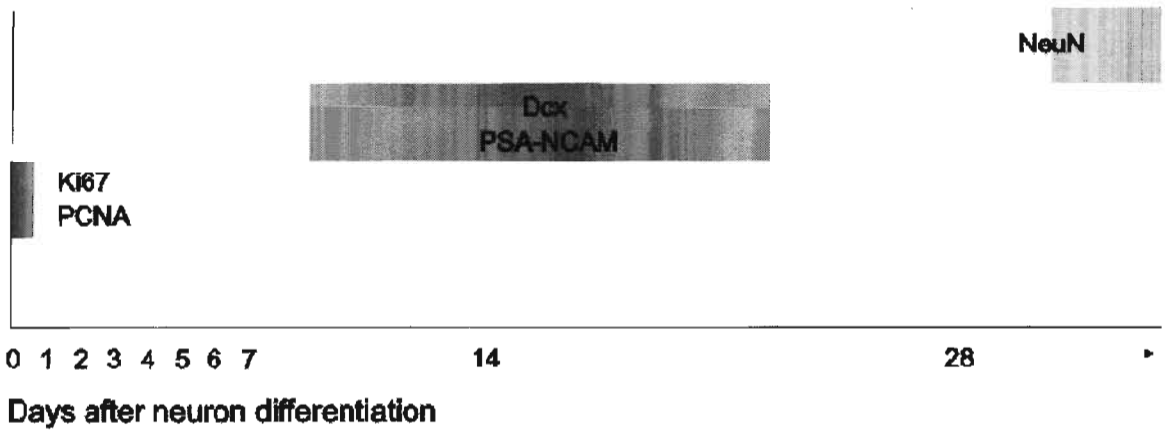

Figure 3. Marker expression pattern of neurons over the course of their development. Proliferative markers, such as Ki67 and PCNA are expressed during the cell cycle. Markers of immature neurons, such as Dcx and PSA-NCAM are expressed until neurons reach maturity, at which time markers of mature neurons (for instance, NeuN) are expressed. Adapted from Zhao et al., (2008).

Functional Significance of New Hippocampal Neurons

The involvement of the hippocampus in learning and memory is a well-established finding (see reviews by Leuner et al., 2006; Zhao et al., 2008). Learning and memory impairments have been attributed specifically to deficits in, or loss of postnatal neurogenesis in the DG. Using various manipulations known to decrease neurogenesis, researchers have shown that hippocampal-dependent memory (specifically spatial

memory in rodents) is also impaired. For example, Thuret et al. (2009) demonstrated that MRL/MpJ mice, which have naturally low rates of neurogenesis compared to C57BL/6 mice, performed worse on a spatial location task than their C57BL/6 counterparts. Similarly, long-term spatial memory was vastly impaired in rats (Snyder et al., 2005; Madsen et al., 2003) and mice (Rola et al., 2004) after low-dose irradiation, which largely blocks neurogenesis while leaving mature cells intact. Furthermore, reduction of neurogenesis by chronic stress (McCormick et al., 2010) and a cytotoxic agent (Goodman et al., 2010) correlated with spatial memory impairments.

In addition to its possible role in learning and memory, neurogenesis may also play a role in mood regulation. Specifically, decreased neurogenesis has been implicated as an underlying cause of depression and post-traumatic stress disorder (PTSD). The therapeutic effects of anti-depressant drugs remain largely unknown, but research has shown that treatment with anti-depressants increases neurogenesis (Malberg et al., 2000; Czeh et al., 2001). Neurogenesis may also be necessary for detection of novelty (Lemaire et al., 1999; Parkin, 1997) and for anxiety-related behaviours (Bannerman et al., 2002; see reviews by Bannerman et al., 2004; Fanselow & Dong, 2009).

Thus, neurogenesis in a discrete region of the hippocampus appears to play a role in a wide variety of brain functions. Further investigation into the structure of the hippocampus revealed that it may be divided into at least two distinct zones: dorsal and ventral (Thompson et al., 2008). In addition to anatomical differences, heterogeneous gene expression across the septotemporal axis (the name is based on the proximity of

the dorsal and ventral hippocampus to the septum and temporal cortex respectively) of the hippocampus support the idea of functionally distinct zones in the hippocampus (Leonardo et al., 2006; see Figure 4). Similarly, lesion studies have demonstrated that selective dorsal or ventral lesions result in dissociated deficits. Specifically, Bannerman et al. (1999) demonstrated that selective lesions in the dorsal hippocampus resulted in impaired spatial memory, whereas lesions to the ventral hippocampus had no effect on memory. Bannerman et al. (2002) corroborated this finding, and by further investigation discovered that ventral lesions resulted in rats being less anxious than intact rats, suggesting that the ventral hippocampus is responsible for appropriate manifestation of anxiety to stimuli. However, there is also evidence that dorsal and ventral hippocampus regions both contribute to spatial learning, but that the dorsal hippocampus has a more pronounced role than the ventral region (Jung et al., 1994). Therefore, there may be functional heterogeneity across the septotemporal axis of the hippocampus, and the functional implications of hippocampal neurogenesis may depend on its location in the septotemporal extent of the hippocampus.

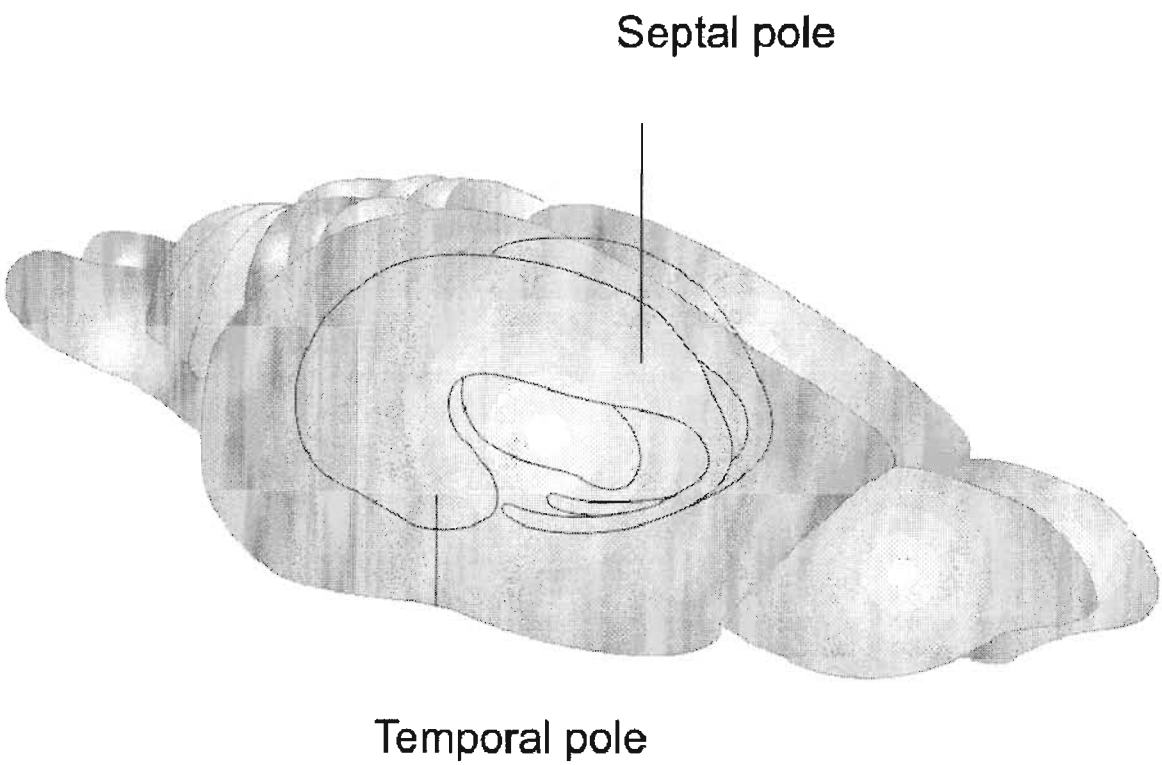

Figure 4. Septotemporal axis of the hippocampus in the rat brain. The septotemporal axis of the hippocampus is named so because of the proximity of the dorsal (septal) and ventral (temporal) poles to the septum and temporal cortex, respectively.

Regulators of Neurogenesis

There are a number of factors that can influence neurogenesis. Although not fully understood, these factors create a complex milieu that can negatively or positively affect neuron proliferation, differentiation, or survival of neurons. For instance, growth and trophic factors, such as brain-derived neurotrophic factor (BDNF; Lee et al., 2002; Scharfman et al., 2005; Sairanen et al., 2005) and vascular endothelial growth factor (VEGF; Rosenstein et al., 2010), have been found to affect neuron proliferation and survival. Similarly, in addition to their conventional roles, some neurotransmitters (e.g. Fricker et al., 2005; Ge et al., 2006) and hormones (reviewed in De Kloet et al., 1998) have been found to affect neurogenesis.

Environmental stimuli can also elicit changes in the proliferation and/or survival of new neurons. For example, learning, environmental enrichment, physical exercise, electroconvulsive shock therapy, selective serotonin reuptake inhibitors (SSRIs), and some disease pathologies enhance cell proliferation and/or survival of newly generated neurons (Ambrogini et al., 2000; Nilsson et al., 1999; van Praag et al., 1999; Helfer et al., 2009; Scott et al., 2000; Malberg et al., 2000; Malberg & Duman, 2003; Warner-Schmidt & Duman, 2006; Czeh et al., 2007; Jin et al., 2003; Mohapel et al., 2004). Conversely, aging, sleep deprivation, radiation, and caffeine have been found to be detrimental to some aspect of neurogenesis (He & Crews, 2007; Heine et al., 2004; Kuhn et al., 1996; Mirescu et al., 2006; Mizumatsu et al., 2003; Rola et al., 2004; Wentz & Magavi, 2009). However, the most studied negative regulator of neurogenesis is stress (Malberg & Duman, 2003; Warner-Schmidt & Duman, 2006; Gould et al., 1998; Westenbroek et al., 2004; Simon et al., 2005; Czeh et al., 2007; Falconer & Galea, 2003; Fornal et al., 2007; McCormick et al., 2010), the focus of this thesis.

Exactly how stress affects neurogenesis has yet to be determined. Nevertheless, it is known that the negative effects of stress are attributed to release of glucocorticoid hormones, particularly CORT. For example, adrenalectomized rats exhibit increased neurogenesis (Cameron & Gould, 1994). Similarly, Tanapat et al. (2001) showed that stress exposure after adrenalectomy resulted in no difference in neurogenesis, whereas animals with intact adrenal glands show decreased neurogenesis in response to the same stressor. Administration of CORT or a synthetic glucocorticoid, dexamethasone, results in decreased cell proliferation (Cameron & Gould, 1994; Kim et al., 2004). CORT

is released as a result of activation of the hypothalamic-pituitary-adrenal (HPA) axis, the main physiological stress response system.

Stress and the HPA Axis

The purpose of the HPA axis is to cope with allostatic load – any factor that threatens homeostasis (reviewed in McEwen, 2008). When a stressful episode is perceived, the immediate response is by the sympathetic nervous system (SNS), which causes increased heart rate and blood pressure, release of catecholamines, and blood glucose levels, generally to prepare the body for survival in the face of stress (reviewed in Ulrich-Lai & Herman, 1996). Next, activation of the HPA axis causes increased secretion of adrenal steroids (particularly glucocorticoid hormones) into the bloodstream. HPA axis activation stimulates the paraventricular nucleus (PVN) of the hypothalamus to release corticotrophin-releasing hormone (CRH). This hormone acts on the anterior pituitary, causing it to release adrenocorticotropic hormone (ACTH) (Whitnall, 1993). ACTH stimulates the adrenal cortex to release CORT, which functions via two similar receptors to regulate its own release by inhibiting CRH and ACTH secretion, in a negative feedback system. Prolonged, elevated levels of CORT have been shown to be damaging. As such, the HPA axis is inherently protective, in that repeated exposure to the same stressor causes a markedly lessened neuroendocrine response, allowing the HPA axis to respond appropriately to new stressors, but limiting the response to previously experienced stressors (Marti & Armario, 1997).

The two receptors for CORT, mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs; Reul & de Kloet, 1985), have differential affinities for

CORT, as well as differential expression in the brain. It is primarily GRs that mediate the stress response (Datson et al., 2008), as MRs have a 10-fold higher affinity for CORT (K_d ~ 0.5 nM) than GRs (K_d ~ 5.0 nM) (de Kloet et al., 2005). Thus MRs are 90% occupied at basal levels of CORT, and GRs are only 10% occupied until a stressor is perceived, when GRs also become occupied. CORT receptors also exist in extrahypothalamic sites, with the highest density in the hippocampus (reviewed in Keller-Wood & Dallman, 1987), which explains why CORT has an effect on neurogenesis.

To recap, stress appears to exerts its negative effect on neurogenesis through the activation of the HPA axis, and subsequently, though actions of CORT. Although the mechanism of how CORT affects neurogenesis is not known, it is hypothesized that CORT is not solely responsible for decreasing neurogenesis. Instead, a concerted effort between CORT and neurotransmitters, particularly glutamate, is proposed (reviewed in McEwen, 2008). Cameron et al. (1995) showed that lesions to the entorhinal cortex, and, thus, removal of glutamatergic afferents, resulted in increased neurogenesis. Similarly, there is evidence that antagonists for the NMDA receptor, a glutamate receptor, increased proliferation of neurons (Gould et al., 1997), and NMDA receptor activation decreased neurogenesis (Cameron et al., 1995). Furthermore, stress-induced CORT secretion is responsible for glutamate accumulation in the hippocampus (Moghaddam et al., 1994; Stein-Behrens et al., 1994; Venero & Borrell, 1999), a state that can result in neuron death by glutamate excitotoxicity.

Stress in Adulthood

Paradigms used in stress research vary in both form and duration. It has been proposed that psychosocial stressors – those that are more relevant to an animal in nature – are more detrimental than laboratory-applied, usually physical stressors (reviewed in McEwen, 2000). Furthermore, both short- and long-term bouts of stress can decrease neurogenesis, although there are some key differences in their effects (discussed below).

Below is a comprehensive review of studies on stress and neurogenesis in the adult male animal, separated by duration and type of stressor (psychosocial, physical, or variable - some combination of the psychosocial and physical stressors). Depending on duration, stressors are classified as acute, subacute, or chronic (lasting hours, a few days, or weeks to months respectively; Sorrells et al., 2009). Whereas studies implementing acute and chronic stressors are common, subacute stressors are few and far between. Because of their scarcity, subacute stressors have been grouped into the chronic stress section.

Chronic Psychosocial Stressors

It has been found that chronic stress in the form of resident-intruder paradigms reduces neurogenesis. Typically, resident-intruder paradigms involve a young, socially-inexperienced animal (the stressed intruder) being introduced into the home cage of an older, dominant animal (the resident). Animals are usually separated for at least part of the time by wire or mesh to avoid physical contact. Czeh et al. (2001) investigated the effect of one week of daily, hourly exposure to a resident-intruder. Decreased cell

proliferation in the hippocampus was detected in stressed tree shrews as measured by BrdU administration on the last day of the experiment. Using a similar protocol for a longer duration of five weeks, Czeh and colleagues (2007) investigated the effects of stress on cell proliferation and survival by administering BrdU at the end of the stress procedure (and euthanizing 24 hours later), or two weeks into the stress procedure (and euthanized 20 days later at termination of the stressor), respectively. They found that chronic stress in rats decreased both proliferation and cell survival. Bartolomucci et al. (2002) and Simon et al. (2005) similarly demonstrated that five weeks of psychosocial conflict followed by a BrdU injection at termination of the experiment to mark cell proliferation, resulted in stressed tree shrews having less neurogenesis than their non-stressed counterparts.

Both Mirescu et al. (2006) and Guzman-Marin et al. (2003) showed that stress in the form of sleep deprivation also decreased neurogenesis. Mirescu et al. (2006) placed adult male Sprague-Dawley rats on small platforms over water to facilitate sleep deprivation (falling asleep resulted in them falling off the platform into the water). Extended sleep deprivation lasting 72 hours (followed by a BrdU injection and euthanasia two hours, one week, or three weeks later to assess cell proliferation, short-term survival, and long-term survival, respectively) resulted in a transient decrease in neurogenesis as a result of short-term stress, as neurogenesis was not significantly different in stress and control groups after three weeks. Guzman-Marin et al. (2003) used a different protocol, in which adult male Sprague-Dawley rats were placed on a treadmill that was intermittently moving for 96 hours. Although there is a slight a

confound in this research, as physical exercise has been shown to increase neurogenesis (van Praag et al. 1999), stressed rats experienced significantly less cell proliferation compared to controls.

Chronic Physical Stressors

Restraint stress, as the name implies, involves physically containing an animal in a confined space. Pham et al. (2003) implemented a chronic restraint stress paradigm, in which adult male Sprague-Dawley rats underwent daily six-hour exposure to the stressor for 21 or 42 days. Cell proliferation and cell survival were measured with BrdU. Twenty-one and 42 days of stress decreased cell proliferation and cell survival, respectively, compared to controls. Also in adult male Sprague-Dawley rats using a similar experimental design (daily six-hour exposure to restraint stress for 14 days), Xu et al. (2006) showed that chronic restraint stress decreased BrdU expression compared to controls, indicative of cell survival in this experiment.

Footshock is another commonly employed physical stressor. In male Sprague-Dawley rats, Shors et al. (2006) demonstrated that footshock administered over seven days for no more than 40 minutes per day was not sufficient to cause a difference in cell proliferation between stress and control rats. In adult male Wistar rats, Westenbroek et al. (2004) looked at the effects of eight days and three weeks of daily footshock on survival of neurons born in the first week of the stress procedure. Eight days of stress had no effect on neurogenesis, but after three weeks of stress, rats exhibited less neurogenesis. However, the results depended heavily on social housing, in that single-housed rats showed decreased neurogenesis, but neurogenesis in socially-housed rats

did not differ from control rats, demonstrating the mediating effects of socialization on the effects of stress.

Chronic Variable Stressors

Heine et al. (2004) looked at the effects of chronic unpredictable stress on neurogenesis in adult male Wistar rats. Over the course of three weeks, rats were exposed to varying durations of cold immobilization, forced swim, restraint, cage crowding, vibration, and isolation, and a BrdU injection was administered at the end of the stress procedure. Rats were allowed to survive for 24 hours or three weeks after termination of the stressor, and it was found that cell proliferation was decreased, as was survival. Toth et al. (2008) used chronic mild stressors (varying durations of overnight illumination, intermittent food and/or water deprivation, cage tilting, and cage mate switching) to study survival of neurons throughout the stress procedure. They found that in adults, neurogenesis was decreased in the DG. In male mice of three different strains, unpredictable chronic mild stress also decreased neurogenesis, although results varied across strain (Mineur et al., 2007). Lee et al. (2006) also used a chronic mild variable stressor, and BrdU injections were administered at the beginning or end of the 19-day stressor to assess the effects of stress on cell proliferation and survival, respectively. Although no effect on cell proliferation was detected, decreased survival was observed in adult male Sprague-Dawley rats.

Acute Psychosocial Stressors

Acute stress has also been found to decrease hippocampal neurogenesis, as demonstrated by Gould et al. (1998). They exposed adult male marmoset monkeys to a

resident-intruder paradigm for one hour, injected the monkeys with BrdU immediately after, and euthanized them two hours later. BrdU immunohistochemistry showed that one hour exposure to a resident, aggressive monkey decreased cell proliferation in the intruder monkeys. Thomas et al. (2007) and Gould et al. (1997) also examined the effects of acute exposure to a resident-intruder paradigm in adult Sprague-Dawley rats and tree shrews, respectively. Thomas et al. (2007) introduced an intruder rat into the home cage of two older, resident rats for 20 minutes, and found that whereas brains of the intruder rats did not differ from controls in cell proliferation or immediate survival, survival was decreased in the short- and long-term. Alternatively, intruder tree shrews in the study by Gould et al. (1997) were exposed to a resident tree shrew for one hour. They found that cell proliferation was decreased in the intruder animals compared to controls.

Tanapat et al. (2001) found that acute exposure to predator odour had an immediate effect on neurogenesis in adult male Sprague-Dawley rats, in that the stressor decreased cell proliferation (two hours after exposure) and immediate survival (one week after exposure). However, the effect was not long lasting, as there was no difference between control and stress conditions three weeks later. Likewise, in males of the same strain, Falconer and Galea (2003) showed that acute exposure to predator odour increased CORT levels and decreased cell proliferation. Conversely, Thomas et al. (2006) demonstrated that a short exposure to predator odour elicited physiological indicators of stress (particularly elevated CORT levels) in male Sprague-Dawley rats, but cell proliferation was unaffected by the stressor.

As described above, prolonged sleep deprivation (Mirescu et al., 2006) resulted in decreased cell proliferation two hours and one week after the stressor, but acute (24 hours) sleep deprivation had no effect on neurogenesis.

Acute Physical Stressors

Adult male Sprague Dawley rats exposed to one hour of restraint stress, injected with BrdU immediately upon relief of the stressor, and perfused two hours later had decreased cell proliferation (Kim et al., 2005). In contrast, there is also evidence that acute stress is not always damaging to neuron generation or survival. As described above, Pham et al. (2003) showed that chronic restraint stress in adult male Sprague-Dawley rats administered daily for 21 or 42 days resulted in decreased cell proliferation, and decreased cell survival compared to controls, respectively. However the same stressor administered as one session of two- or six-hour stress had no effect on cell proliferation in the DG.

Adult Sprague-Dawley rats exposed to shock stress produced variable results. Fornal et al. (2007) assessed whether one session of 100 tail shocks would affect cell proliferation. By administering BrdU one hour, two hours, or seven days after the stress exposure, they found that despite CORT increase, tail shock had no effect on cell proliferation. Conversely, Shors et al. (2006) showed that a single footshock session decreased cell proliferation in male Sprague-Dawley rats. Furthermore, Malberg and Duman (2003) showed that short-term exposure to footshock resulted in decreased proliferation in male rats.

Summary

The studies described above indicate that chronic stress in adulthood in male animals reliably decreases neurogenesis, but the effects of acute stress are more variable. These results are somewhat perplexing, as elevated glucocorticoid levels are thought to be damaging to neurogenesis. The work by Thomas et al. (2006) and Pham et al. (2003) are at least two examples of stress increasing CORT levels, but sparing neurogenesis. Therefore, a direct, inverse relationship between CORT and neurogenesis may not be as simple as previously described. It may be that, when faced with short-lived, mild stressors, the HPA axis can respond appropriately and maintain homeostasis through adaptation. However, severe or chronic stress (constituting allostatic overload), can dysregulate the HPA axis and lead to damaging effects, including decreased neurogenesis. Furthermore, stress-induced decline in adult neurogenesis may not be long-lasting, as demonstrated by Heine et al. (2004), and Mirescu et al. (2006). Both groups showed that chronic stress decreased neurogenesis, but the effects were reversed within three weeks. Therefore, the effects of stress in adulthood may be transient.

As noted above, most studies examining the effects of stress on neurogenesis have used male, adult rats. The few experiments involving female animals have been omitted from the above literature review as the effects of stress on neurogenesis observed in females are more variable than those observed in males. For instance, whereas Falconer and Galea (2003) found that in male rats, acute exposure to predator odour increased CORT levels and decreased cell proliferation, the stressor had no effect

on cell proliferation in females despite elevated CORT levels. Similarly, Shors et al. (2006) showed that a single footshock session decreased cell proliferation in male, but not female rats. Westenbroek et al. (2004) also found differing effects of stress in male and female rats, in that males housed individually or in social groups experienced decreased, or no change in neurogenesis, respectively. However, regardless of housing, female rats showed increased neurogenesis in response to stress. Sex hormones may account for the observed sex differences, but the involvement of sex hormones is outside the scope of this thesis, and therefore the focus of this review is on studies involving male animals.

As demonstrated above, the effects of stress in adult male rats have been thoroughly studied, but the effect of stress during adolescence has not been as widely researched, despite the fact that adolescence is a sensitive period of brain development (McCormick et al., 2007; see reviews by McCormick & Mathews, 2007; Crews et al., 2007).

Adolescence

The temporal definition of adolescence in rats varies, largely because of its lack of a defining beginning or end. Weaning usually occurs on PND 21, and from PND 60 on is considered to be adulthood. However, there is debate over whether the interim – adolescence – should be defined in relation to physical growth, puberty, behaviour, or other aspects of maturity (reviewed in Spear, 2000). In this thesis early adolescence is defined as PND 21 to 34, mid-adolescence as PND 34 to 46, and late adolescence as PND

46 to 59, with puberty occurring therein (Tirelli et al., 2003). However, others have defined adolescence more conservatively, from PND 28 to 42 (Spear, 2000).

During adolescence, the brain is still undergoing much development. The hippocampus in particular undergoes various morphological changes, including noteworthy synaptic pruning (Swann et al., 1999) and development of neurotransmitter systems (reviewed in Crews et al., 2007). These developmental changes are thought to be the underlying cause of a set of behaviours that are specific to adolescent rats, including high levels of risk-taking, novelty-seeking, exploration, play behaviour and sociability (see reviews by Spear, 2000; McCormick & Mathews, 2007; Crews et al., 2007).

It seems that stress affects adolescents more profoundly than adults (reviewed in Spear, 2000). Five days of chronic restraint or social stress (one hour restraint or five minutes per day exposure to an isolated adult male, respectively) in male mice differentially affected adolescent and adult mice. Both stressors decreased body weight in the adolescent but not adult animals, demonstrating that adolescents are more susceptible to the negative effects of stress (Stone & Quartermain, 1997). This may be explained by the fact that the adolescent rat shows alterations in stress reactivity compared to adults, thought to be the result of HPA axis immaturity. This is exemplified by a prolonged release of CORT that has been observed in response to acute stress (Goldman et al., 1973; Walker et al., 2001; Romeo et al., 2006), and by a higher CORT response but faster return to baseline in response to chronic stress (Romeo et al., 2006).

However, most relevant to the topic of stress and neurogenesis, is that the amount of neurogenesis is much higher during adolescence than during adulthood. McDonald and Wojtowicz (2005) demonstrated that from 38-days to 12-months of age, rats experience a 94% reduction in neurogenesis. How the high rate of neurogenesis around PND 38 is affected by stress is not known, mainly because there are very few studies looking at the effects of stress on neurogenesis during adolescence, and the ones that exist are vastly contradictory.

Stress in Adolescence

There are very few studies investigating the effects of stress on neuron generation during adolescence. However, there are a number of studies examining the effects of stress on other measures. For instance, adolescent stress exposure has been found to decrease hippocampal volume, cause both immediate and long-lasting deficits in spatial memory (Isgor et al., 2004; Sterlemann et al., 2009), and alter both normal and drug-induced behavioural responses (Stone & Quartermain, 1997; Mathews et al., 2008).

McCormick et al. (2010) previously reported that adolescent female Long Evans rats exposed to chronic mild social stress (social isolation and instability stress lasting 16 days) caused the rats to gain less weight than the controls, and decreased neurogenesis, suggesting that like in adults, chronic stress decreases neurogenesis. However, Toth et al. (2008) used a variable chronic mild stressor (described above) to compare the effects of stress on neuron survival in adolescent and adult male Sprague-Dawley rats. Whereas chronic stress decreased the survival of neurons in adult rats, the same

stressor increased neurogenesis in adolescent rats, while CORT was increased in both age groups during the stressor. Barha et al. (in press) investigated the effects of chronic intermittent stress on male and female Sprague-Dawley rats. Their stressor consisted of one hour of restraint stress every other day for three weeks during adolescence. Rats were injected with BrdU roughly 3 weeks later in adulthood (PND 70), and rats were euthanized 3 weeks post-injection. Both proliferation and survival of cells in the DG were decreased in females, and slightly increased in male rats. The majority of surviving cells were in fact neurons, as more than 80% of surviving cells were co-labeled with NeuN.

Therefore, more work needs to be done to understand how stress affects neurogenesis during this critical period of brain development. Importantly, a number of commonalities exist between the few studies on adolescent stress. First, opposite to the effects of stress during adulthood, neurogenesis is increased in adolescent male rats, and decreased in females (Barha et al., in press; McCormick et al., 2010). Second, the effects of adolescent stress and neurogenesis seem to be long-lasting. It is possible that stress during adolescence may be rewiring the hippocampus, causing alterations in neurogenesis that last into adulthood. Accordingly, the effects of stress during adolescence may underlie adult-onset behavioural abnormalities, such as mood and anxiety disorders, and other psychopathologies (Tsoory et al., 2008).

Current Study

The rationale for the current study was the scarcity of research on the effects of stress administered during adolescence on neurogenesis. The aim of the current study

was to investigate my hypothesis, that chronic, mild social stress will decrease neurogenesis during adolescence, as has been found numerous times in adults, and that any effect observed would be long-lasting.

This hypothesis was based on a number of observations. The paradigm of chronic mild stress used in the current study is a combination of social isolation and social instability, found numerous times to be successful at evoking increased CORT level and decreasing weight gain in adolescent rats (McCormick et al., 2007; 2008; 2010; reviewed in McCormick et al., 2010). The social isolation aspect of the stressor was found to be successful at evoking elevations in CORT release, but the instability stress, which consisted of introduction of rats to a new cage partner daily over the course of the stressor, prolonged the high level of CORT elicited by the social isolation (McCormick et al., 2007). It is, therefore, thought that the social instability aspect of the stressor decreases the chances of rats habituating to the social isolation phase of the stressor. The stressor was also designed to be specifically stressful to the adolescent rat, based on a few key details. As previously discussed, adolescent rats are more social than adults (reviewed in Spear, 2000), and social bonds formed during adolescence are important for mediating the adverse effects of stress (Terranova et al., 1999). Along the same lines, social experiences are more rewarding to adolescent rats compared to adults (Douglas et al., 2004; reviewed in McCormick, 2010), and thus social isolation and instability should be more stressful to adolescent rats. Accordingly, it was predicted that the social stressor used here would cause a marked decrease in neurogenesis in adolescent male rats.

Materials and Methods

Materials

Please see Appendix A for a list of materials used.

Methods

Animals

Male Long Evans rats (Charles River, St. Constant, QC) were weaned on PND21, and arrived at the colony on PND22. Animals had access to food and water *ad libitum*, and were housed in same-sex pairs on a 12 hour light-dark cycle, with lights on at 8:00 am. All efforts were made to limit the number of animals needed to complete this study, and all animal research was conducted in accordance with the guidelines set by the Canadian Council on Animal Care (CCAC). The protocol was approved by the Brock University Animal Care and Use Committee (ACUC).

Stress paradigm

After one week acclimatization to the colony, rats in all experiments were randomly assigned to the non-stress control (CTL) condition, or were subjected to our adolescent social instability stress protocol (McCormick et al., 2004; 2007; 2010). Rats were either exposed to the stressor chronically for 16 days, constituting the chronic social stress (CSS) condition, or in some experiments, subacutely (SubSS) for 3 days. From PND 30 – 45 (CSS) or from PND 30 – 32 (SubSS), rats were isolated from their cage partners and the colony at varying times throughout the light cycle, for one hour per day. Isolation consisted of animals being placed in ventilated plastic containers

measuring 10 cm in height and 14 cm in diameter. Upon return to the colony, SubSS and CSS rats were placed in new cages and paired with a novel cage partner every day for 16 days, after which they remained with their original cage partners. CTL rats remained with their original cage partners and were disturbed only for weighing and regular cage maintenance.

Experimental Procedures

Experiment 1 – BrdU Injections

To study neurogenesis, both exogenous and endogenous labels were used. For experiment 1, BrdU was administered to mark neurogenesis (Figure 5). BrdU is incorporated only into the DNA of dividing cells in the synthesis phase (S phase) of the cell cycle (Miller & Nowakowski, 1988), and is bioavailable for two hours after injection (McDonald & Wojtowicz, 2005). The saturating dose of BrdU required to mark all neurogenesis in adult rats is reportedly 300 mg/kg (Cameron & McKay, 2001; Hancock et al., 2009). This dose is simultaneously a high dose found to be free of adverse consequences (Cameron & McKay, 2001). However, as previously mentioned, neurogenesis in adolescence is much higher in adolescence compared to adulthood (McDonald & Wojtowicz, 2005; He & Crews, 2007). Therefore, in an attempt to mark maximal cell generation in adolescent rats, I chose to employ a multiple injection protocol, in which the rats received one 300 mg/kg injection of BrdU daily for three consecutive days. Because BrdU is an indiscriminate marker of new cell generation (both glia and neurons), the endogenous marker Dcx was also used to determine if stress affected neuron survival in particular. Dcx is a microtubule associated-protein,

found to be expressed exclusively in migrating neuroblasts, and is therefore an indicator of developing neurons (Brown et al., 2003; Couillard-Depres et al., 2005). Although little is known about the function of Dcx, it is clear that it is required for normal neuronal migration (Gleeson et al., 1999). Compared to the small window of availability for BrdU incorporation, Dcx is expressed for a much larger window, as it is expressed for roughly three weeks while neurons are still immature (Figure 6).

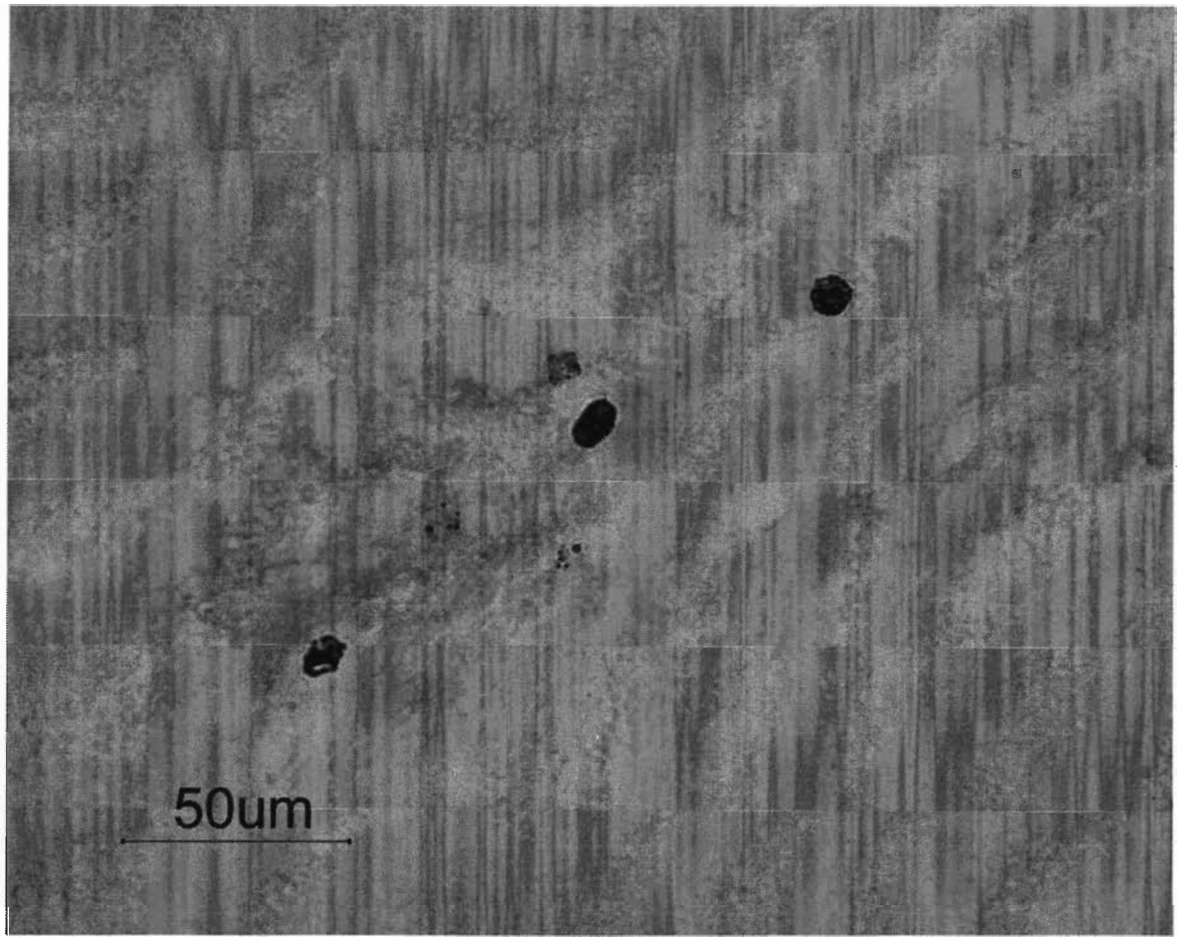

Figure 5. Representative image of BrdU-ir cells, viewed under 400x magnification. BrdU is incorporated into the nuclei cells undergoing DNA replication in the S phase. BrdU remains in circulation, available to be incorporated for 2 hours following injection. BrdU staining can be either uniform or punctuate, as this image shows.

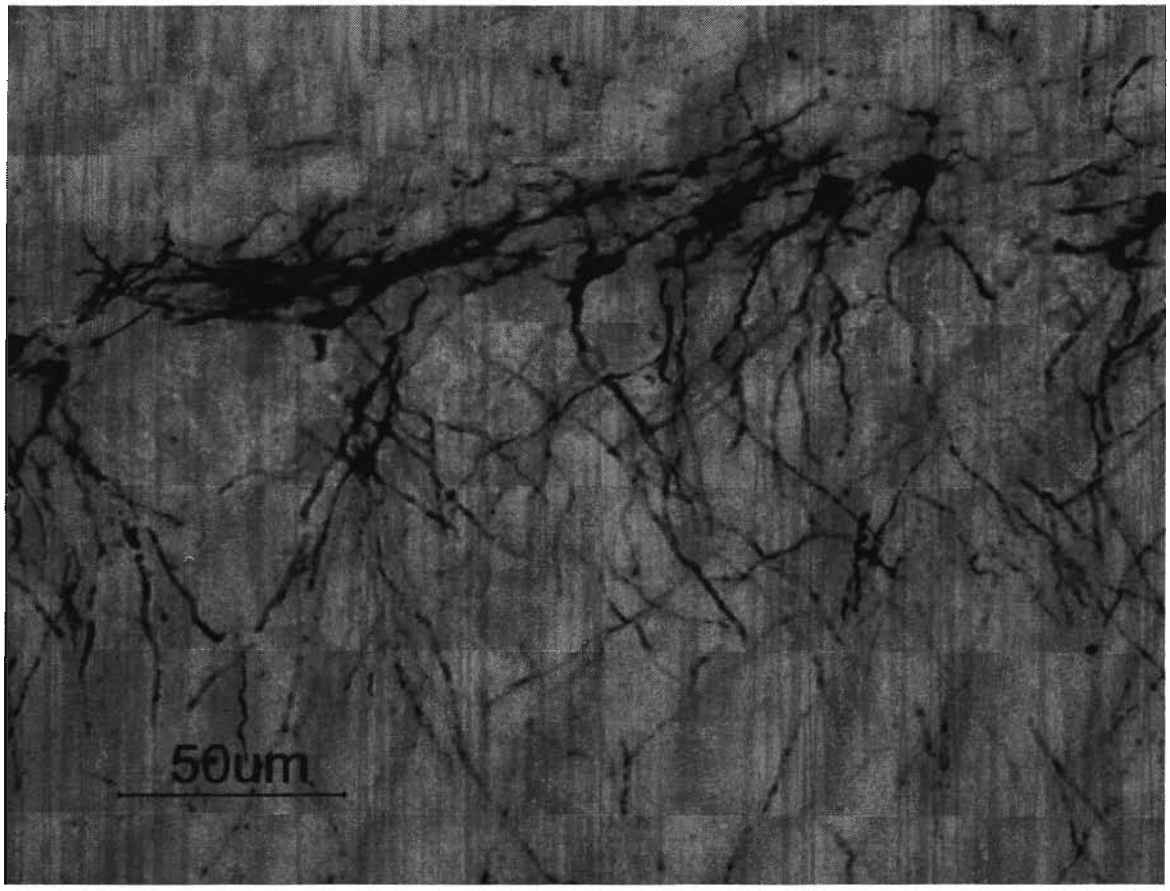

Figure 6. Representative image of Dcx-ir cells viewed under 400x magnification. Dcx is a marker of immature neurons, and is expressed in newborn neurons that are a few days old until they start expressing markers of mature neurons, roughly 3 weeks later. Dcx-ir cells are located in the SGZ and GCL, depending on the stage of maturation they are in. Naïve cells are located in the SGZ, and do not exhibit projections, whereas those later in development migrate into the GCL, and project extensions through the GCL into the ML.

Experiment 1a: Rats were stressed as described above, randomly assigned to the SubSS, CSS, and CTL conditions (n = 40) and administered daily intraperitoneal (ip) injections of 5-Bromo-2'-deoxyuridine (BrdU; 300 mg/kg, Sigma) on PND 30 – 32 to measure the effects of social stress on the cells generated at the beginning of the stress procedure. Rats were euthanized 24 hours after the last BrdU injection (PND 33) to examine the effects of social stress on proliferation, or two weeks (PND 46) after the last injection to measure the effects of stress on cell survival (see Figure 7).

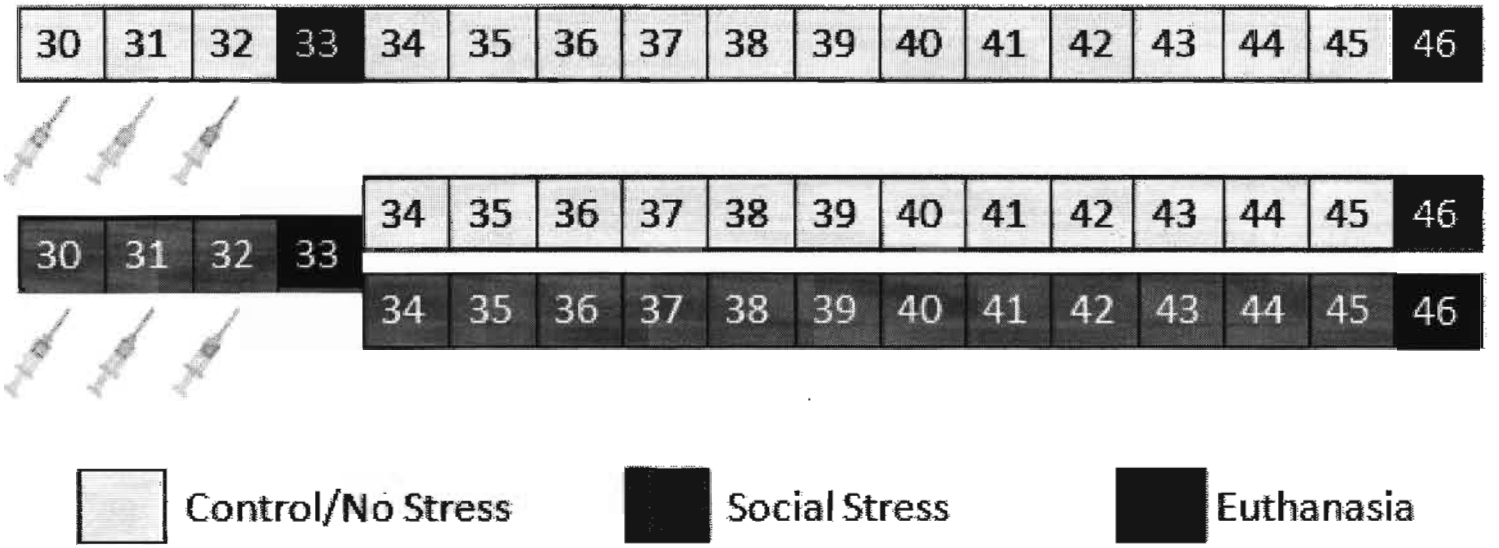

Figure 7. Experimental Design for Experiment 1a. CTL rats remained undisturbed except for regular cage maintenance and weighing, whereas the CSS rats experienced the social stress procedure from PND 30 to 45. One group of rats (SubSS) was only stressed for three days (PND 30, 31, and 32) to assess the effects of subacute stress compared to chronic stress. All rats were injected with BrdU for three consecutive days, starting on PND 30. Rats were either euthanized 24 hours after the last BrdU injection (PND 33) or 24 hours after the last day of stress (PND 46). The effects of both subacute and chronic stress on the cells born at the beginning of the stress procedure were examined using BrdU and Dcx immunohistochemistry.

Experiment 1b: Rats were stressed as described above, randomly assigned to the CSS and CTL conditions (n = 32) and administered daily BrdU (ip) injections (300 mg/kg) on PND 46 – 48 to measure the effects of social stress after the stress procedure. Rats were euthanized 24 hours or four weeks after the last BrdU injection (PND 49 and 75) to measure the effects of social stress on proliferation, and cell survival, respectively (see Figure 8).

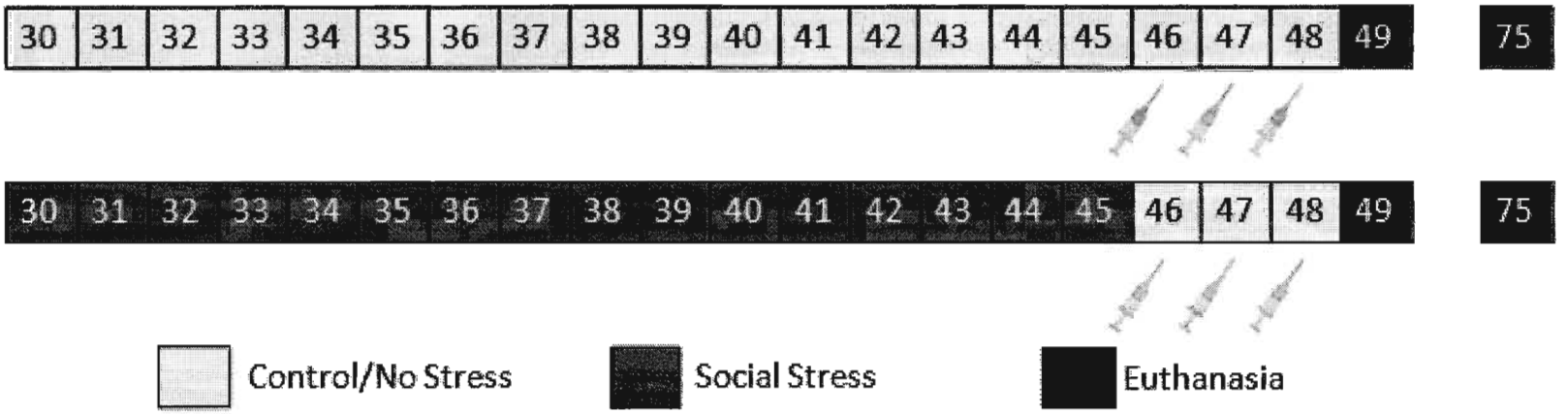

Figure 8. Experimental Design for Experimental 1b. CTL rats remained undisturbed except for regular cage maintenance and weighing, whereas the CSS rats experienced the social stress procedure from PND 30 to 45. All rats were injected with BrdU for three consecutive days, start on PND 46. Rats were either euthanized 24 hours (PND 49), or one month (PND 75) after the last BrdU injection. The effect of chronic stress on the cells born after the stress procedure was examined using BrdU and Dcx immunohistochemistry.

Experiment 2 – Endogenous labels

For experiment 2, I investigated the effects of stress on neurogenesis using only endogenous labels to study cell proliferation and survival. In experiment 2a, I investigated the effects of stress at three different euthanasia time points (PND 33, 46 and 75), and in experiment 2b I examined whether behavioural testing (behavioural data not reported here, see Appendix B) impacted any observed stress effect on neurogenesis. Instead of BrdU, to mark general cell proliferation, Ki67 was used (Figure 9). Ki67 is a nucleus-associated protein of undetermined significance, but its expression is closely associated with all active phases of the cell cycle (G_1, S,G_2, and mitosis; Scholzen & Gerdes, 2000), and thus reflects proliferative events of all new cells during the last 24 hours of an animal's life (reviewed in Becker & Wojtowicz, 2006). Because Ki67 is an endogenous protein, it does not have any of the possible adverse side effects like BrdU (Kee et al., 2002). Dcx was again used to identify neural fate.

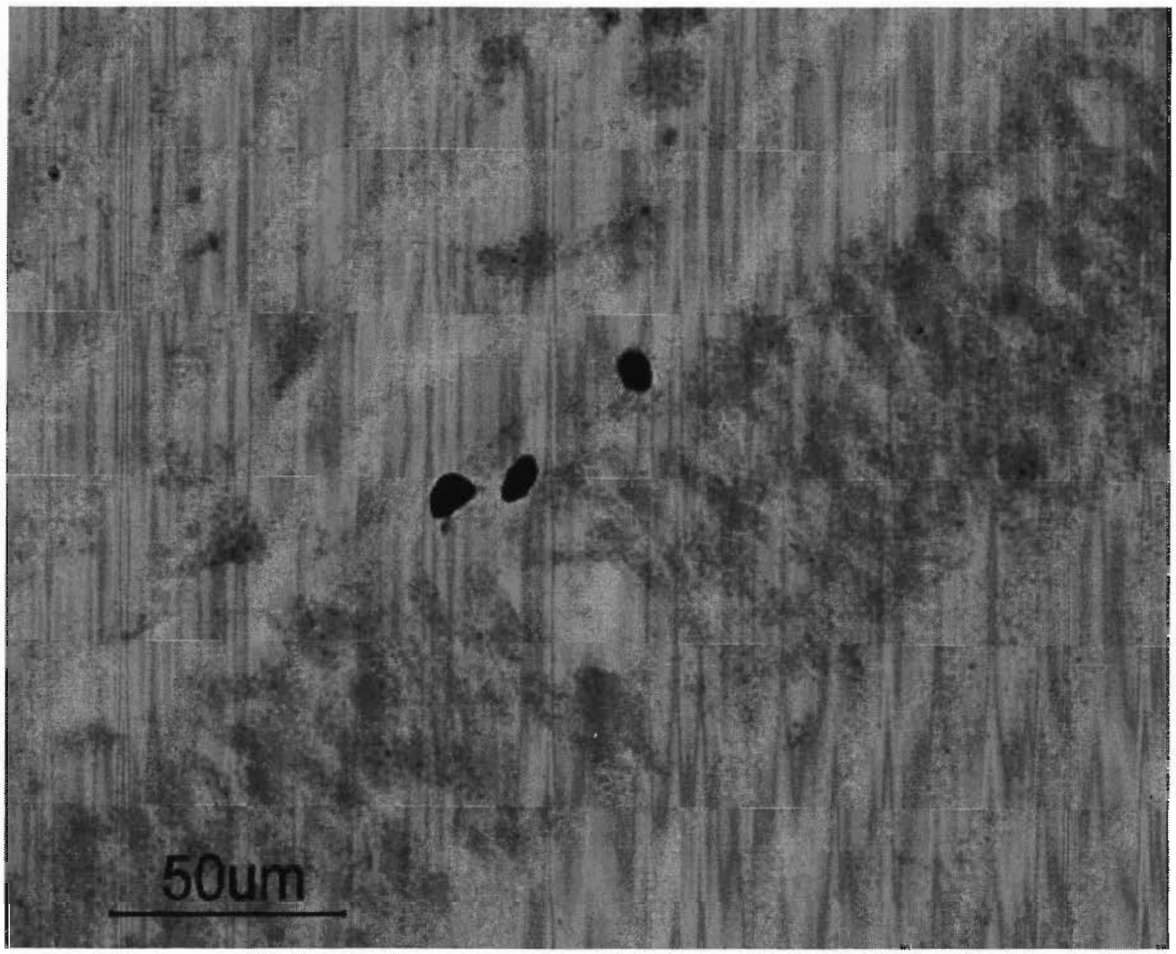

Figure 9. Representative image of Ki67-ir cells, viewed under 400x magnification. Ki67 antibodies detect the Ki67 nuclear protein of all proliferating cells (those in G_1, S, G_2, or M phase of the cell cycle) up to 24-hours before euthanasia of an animal (as the adult cell cycle lasts roughly 24 hours, all proliferative events happening at the time of the animal's death are detected using Ki67 antibodies). Ki67-ir cells are exclusively located in the SGZ, the interface between the GCL and hilus.

Recent evidence has suggested that activation of the resident immune cells of the brain, microglia, can negatively affect neurogenesis (reviewed in Ekdahl et al., 2009). Accordingly, in Experiments 2a and 2b, I sought to add to the limited research on the topic, and investigate the effects of stress on microglia. Microglia are formed from macrophages that originate in the bone marrow and migrate into the brain, where they differentiate into microglia (Streit et al., 2005). In general, infection, invasion by foreign bodies, or injury can result in inflammation, which in the brain, results in activation of otherwise resting microglia. Furthermore, stress-induced HPA axis activation has been found to affect microglia, as they are equipped to respond to CORT, and express both GRs and MRs (Tanaka et al., 1997). There has been much debate over whether the effector molecule of the HPA axis, CORT, stimulates a pro- or anti-inflammatory response from the immune system which seems to be contingent upon the region of the brain that is examined (reviewed in Sorrells et al., 2009). Regardless, in addition to the stress and the HPA axis separately affecting neurogenesis and microglial activation, it has been demonstrated that activated microglia and ensuing pro-inflammatory responses are detrimental to neurogenesis in the adult brain. Ekdahl et al. (2003) introduced adult male Sprague-Dawley rats to four weeks of intracortical delivery of lipopolysaccaride (LPS) or vehicle. LPS infusion significantly increased the number of active microglia and decreased the number of new neurons (shown in BrdU and NeuN double-labeled cells). Similarly, systemic delivery of LPS to adult female rats (followed by six days of BrdU injection and euthanasia the next day) increased the density of

activated microglia cells specifically in the DG, and decreased neurogenesis, as reflected by cells double-labeled with BrdU and Dcx (Monje et al., 2003).

Specifically, I sought to determine if our model of chronic mild stress affected the number of total (resting) or activated microglia. Specifically, OX-42 recognizes protein CD11b, which is part of complement receptor 3 (CR3), expressed on the surface of all macrophages (Rana et al., 2010). When macrophages (in the brain, microglia) become activated, the expression of CR3 becomes highly expressed. Accordingly, I used OX-42, a marker of both quiescent and activated microglia (Rana et al., 2010) to investigate whether our stressor had an effect on the microglia population in the DG, as indicated by the total number of microglia and the number of activated microglia. It was possible to differentiate between the two cell forms because they are morphologically distinguishable (Figure 10). Resting microglia have a ramified appearance, with thin, long processes, whereas activated microglia have few thick, short processes and large cell bodies (reviewed in Mathieu et al., 2010).

Experiment 2a: Rats were assigned to the SubSS, CSS, and CTL groups (n = 48) as described above and were euthanized either on PND 33, 46 or 75 to look at the immediate and long-lasting effects of stress using endogenous markers (Ki67, Dcx, and OX-42) rather than BrdU as in Experiment 1 (see Figure 11).

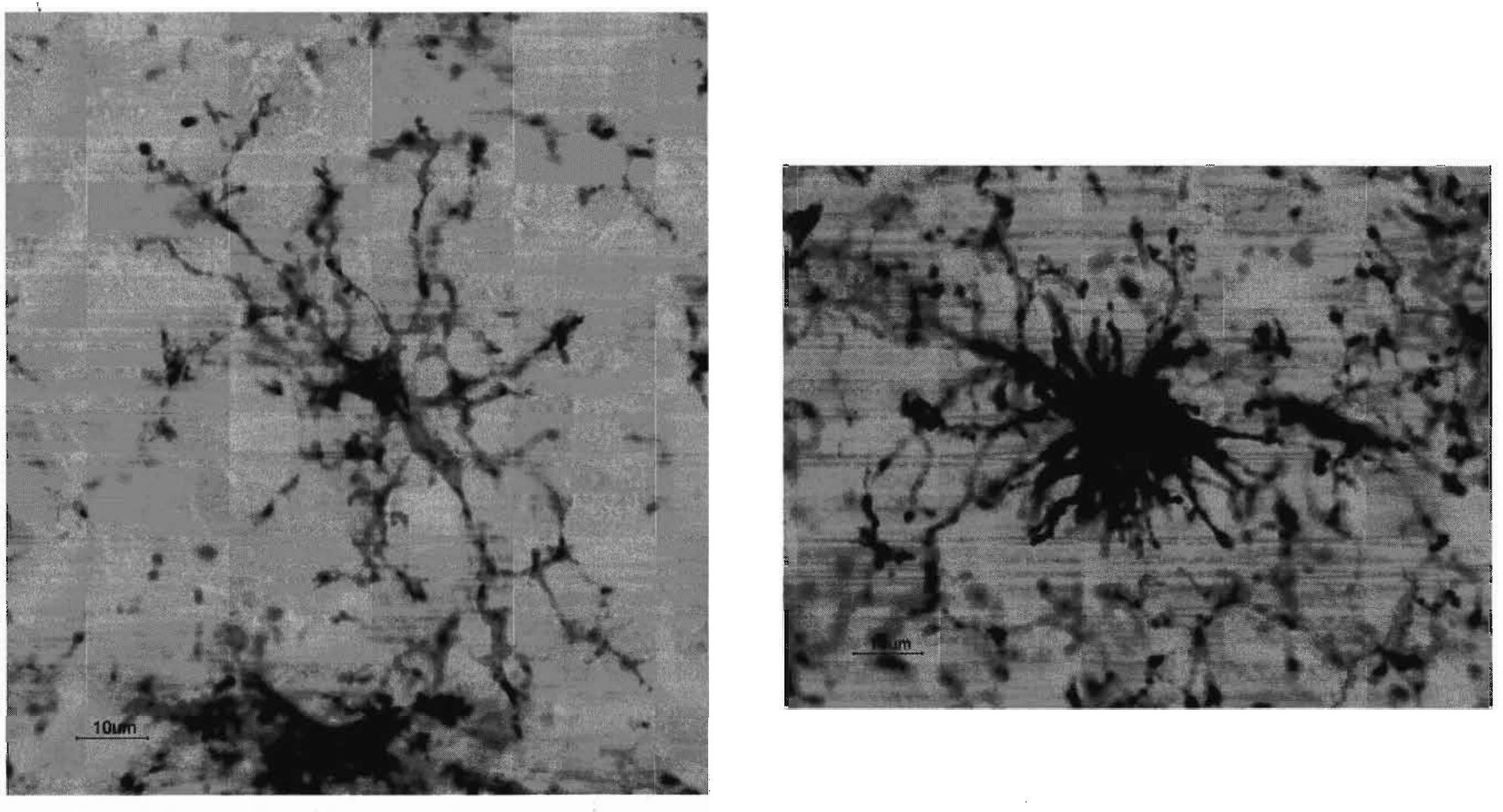

Figure 10. Representative image of OX-42-ir cells viewed under 1000x magnification. OX-42 is a marker of all macrophages, and in the brain marks all microglia cells. Non-activated (left) and activated (right) can be distinguished by their differences in morphology. Non-activated cells have long, thin processes, whereas activated microglia have thick, short processes. Microglia are ubiquitously located throughout the brain.

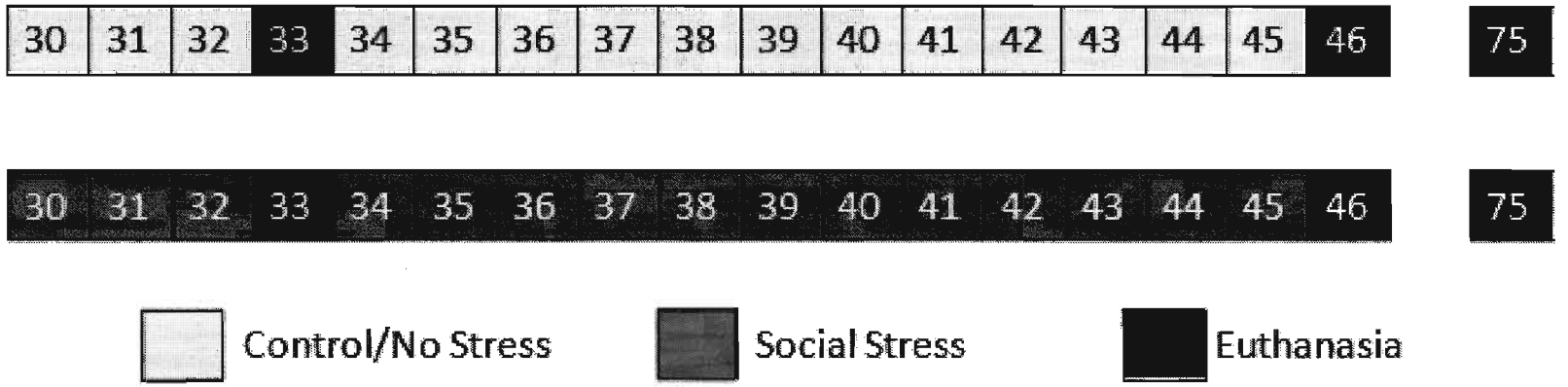

Figure 11. Experimental design for experiment 2a. CTL rats remained undisturbed except for regular cage maintenance and weighing, whereas the CSS rats experienced the social stress procedure from PND 30 to 45. Three euthanasia time points, PND 33, 46, and 75 were selected, and Ki67, Dcx, and OX-42 immunohistochemistry was performed. Thus, the effects of the initial phase of the stressor (PND 33), immediately after the stressor (PND 46), and a month after the stressor (PND 75) on neurogenesis and the immune system were examined.

Experiment 2b: Another group of rats was assigned to the CSS, and CTL groups (n = 20) as above, but underwent behavioural testing (not reported in this thesis but see Appendix B) on PND 46 – 47 and again on PND 70 – 71 (Experiment 2b). The stress procedure and behavioural testing of rats in Experiment 2b were done by Cheryl Sheridan and Feather Nixon. These animals were euthanized on PND 74 to investigate whether or not any long-lasting effects of the stress procedure were altered by subsequent behavioural testing (see Figure 12). The endogenous markers, Ki67, Dcx, and OX-42 were used to accomplish this.

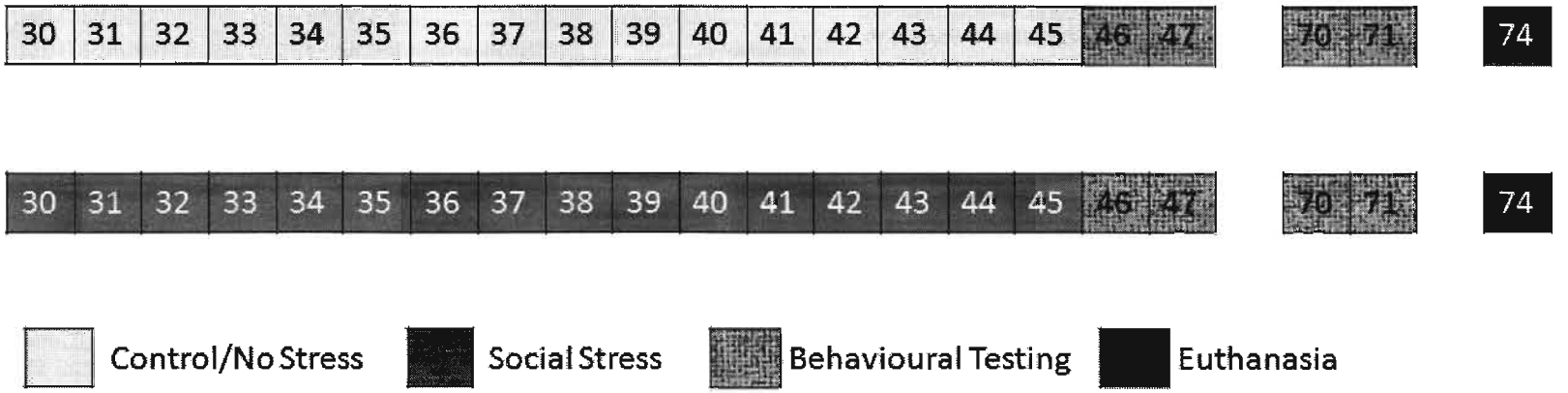

Figure 12. Experimental design for experiment 2b. CTL rats remained undisturbed except for regular cage maintenance and weighing, whereas the CSS rats experienced the social stress procedure from PND 30 to 45. Rats underwent behavioural testing on PND 46 – 47 and again on PND 70 – 71. Rats were euthanized on PND 74, and Ki67, Dcx, and OX-42 immunohistochemistry was performed. This experiment was included to identify if the effects of stress on neurogenesis were altered by behavioural testing.

Histology

Rats were deeply anaesthetized with Euthanyl™ (sodium pentobarbital), and transcardially perfused with 0.9% saline, then 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Brains were removed and post-fixed in 30% sucrose in 4% paraformaldehyde for 2-3 days at 4°C, until equilibrated. Brains were then sliced coronally using a cryostat to obtain 40 µm thick sections, with collection starting at the most anterior region of the DG of the hippocampus, and ending at the most posterior region of the DG. Every 12th section was collected, resulting in eight to ten slices collected per rat. Because the rat brain has not reached full size during adolescence (Isgor et al., 2004), the rats euthanized on PND 33 tended to have slightly smaller brains, and accordingly an average of eight sections were collected, whereas the older rats (euthanized on PND 75) had full-sized brains, and yielded an average of 10 sections. Sections were stored and frozen in cryoprotectant until immunohistochemistry was performed.

Immunohistochemistry

To detect BrdU, free-floating sections were first denatured in 2N HCl for 30 minutes, and to detect Ki67, sections were first incubated at 80°C in 10 mM sodium citrate buffer, pH 6 for 30 minutes. Protocols continued with (and in the case of doublecortin (Dcx) and OX-42 immunohistochemistry, began with) the treatment of 0.3% H_2O_2 to quench endogenous peroxidases. With intermittent washes in PBS-X (0.1 M PBS and 0.3% Triton X-100) throughout, non-specific binding was blocked in 10% blocking solution (0.1 M PBS, 0.3% Triton X-100, and 10% normal serum) for 90 minutes. Sections were then incubated for 24 hours with primary antibody at room temperature.

The following primary antibodies and dilutions were used: rat anti-BrdU (1:200, Accurate Chemical & Scientific Corporation), goat anti-Dcx (1:1000, Santa Cruz Biotechnology), rabbit anti-Ki67 (1:5000, Vector Laboratories), and the OX-42 antibody, mouse anti-rat CD11b (1:10 000, AbD Serotec). Sections were then incubated with secondary antibody for 2 hours at room temperature. The following secondary antibodies were used: biotinylated anti-rat IgG (1:950, Vector Laboratories), horse anti-goat IgG (1:500, Vector Laboratories), goat anti-rabbit IgG (1:500, Vector Laboratories), and horse anti-mouse IgG (1:750, Vector Laboratories). Sections were subsequently incubated with Elite Vectastain avidin-biotin complex (ABC) for 45 minutes, and for antigen retrieval, they were exposed to either diaminobenzidine (DAB) or Very Intense Purple (VIP; Vector Laboratories) for roughly 5 minutes. Sections were then mounted onto slides and taken through a dehydration series of 70, 95, and 100% ethanol for 3 minutes each to dehydrate the sections, followed by at least 3 minutes in xylene. Slides were then cover-slipped using Permount™.

Microscopy

Slides were analyzed using a Nikon Eclipse 80i microscope, equipped with a Nikon DXM1200F digital camera. The experimenter was blind to the experimental groups in all cases. The numbers of cells immunoreactive (ir) for BrdU, Ki67, Dcx, and OX-42 were counted on separate series of sections under 400x magnification. For all markers, cells located in the subgranular zone (SGZ) and granule cell layer (GCL) of the DG of all hemispheres were counted, and were separated into dorsal and ventral groups as defined by Thompson et al. (2008), and Fanselow and Dong (2009). Briefly, the

hippocampus is banana-shaped – its dorsal, anterior portion lies adjacent to the septum, and terminates ventrally near the temporal cortex (Amaral et al. 2007). Generally, I defined the dorsal DG as spanning Figures 48 – 76 of the rat atlas, and the ventral DG spanning from Figure 76 to 89 (Paxinos & Watson, 2005). For each immunohistochemistry assay, the number of dorsal and ventral cells was summed separately, corrected for the total number of hippocampal slices counted, and multiplied by 12 to reflect an estimate for the total number of immunoreactive cells in the DG. Only rats with an adequate number of sections (five sections or more) were included in counting and subsequent statistical analysis.

Cells were classified as "stained" or "not stained." The degree of staining was not scored. Activated microglia were differentiated from non-activated microglia based on morphological differences. Mathieu et al. (2010) defined four stages, during which non-activated cells become fully activated. I used this classification, simplified to classify cells as non-activated (small cell bodies with long, thin processes) or activated (large, round cell bodies with or without short, thick processes, as seen in Figure 10).

Results

Statistical analysis was performed using SPSS to identify differences in immunoreactive cell counts. Two or three factor Analysis of Variance (ANOVA), t-tests, and post hoc t-tests or F tests for simple effects were performed where appropriate. To reiterate, SubSS, CSS, and CTL stand for subacute social stress, chronic social stress, and control rats, respectively. Statistical significance was defined as an alpha level of $p < 0.05$.

Experiment 1 – BrdU Injections

Experiment 1a – Investigating the effects of SubSS and CSS on hippocampal neurogenesis born at the beginning of the stress procedures, using BrdU and Dcx

BrdU: Rats were injected with BrdU on PND 30 – 32 to examine the effects of stress on the cells generated at the beginning of the stress procedure. At PND 33, there was no significant difference between SubSS/CSS and CTL in BrdU-ir ($t_{13} = 0.68$, $p = 0.51$). At PND 46 there was no significant difference among the groups in BrdU-ir cell numbers ($F_{2,19} = 0.33$, $p = 0.73$; see Figure 13). To see if the increase in neurogenesis from PND 33 to 46 was greater for CTL than CSS a t-test for each stress group was performed between cell counts on PND 33 and on PND 46. For CTL rats, the increase was significant ($t_{13} = 2.72$, $p = 0.02$). For CSS rats the difference was not significant ($t_{13} = 1.45$, $p = 0.17$). Similarly, to see if neurogenesis increased in the SubSS rats from PND 33 to 46, a t-test was performed. The increase was significant ($t_{12} = 2.36$, $p = 0.04$).

Dcx: At PND 46, there was no significant difference among the groups in Dcx-ir cell numbers ($F_{2,19} = 0.94$, $p = 0.41$; see Figure 14).

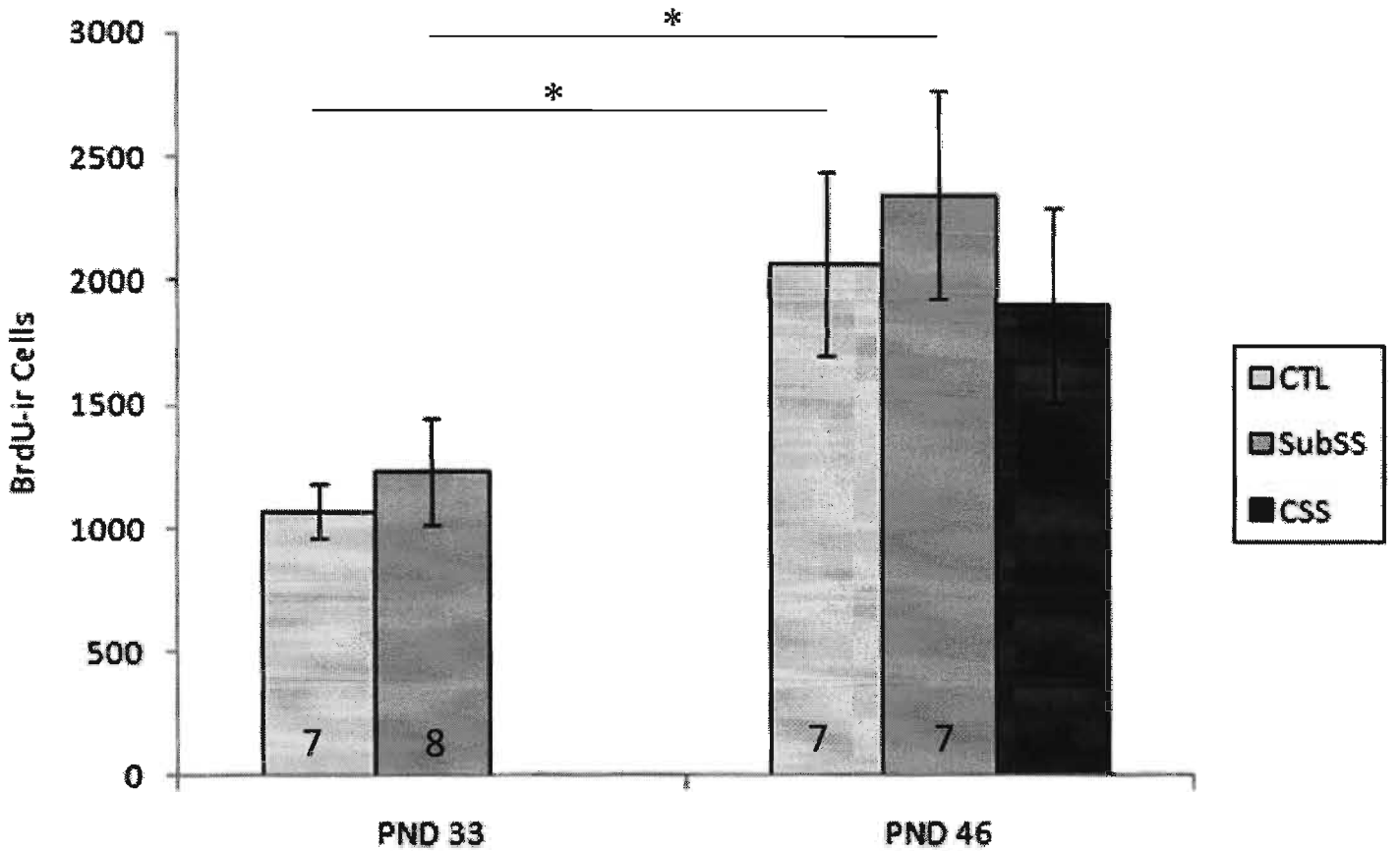

Figure 13. Experiment 1a. Mean number of BrdU-ir cells in the DG of the hippocampus on PND 33 and 46. On PND 33, no significant difference was found between the SubSS/CSS and CTL groups ($p = 0.51$). On PND 46, there was no difference between the groups ($p = 0.73$), but the SS group showed a trend for decreased cell genesis compared to CTL. It was also discovered that the increase in the number of BrdU-ir cells from PND 33 to 46 was significant for the CTL and SubSS groups ($p = 0.02$ and 0.04 respectively), but not the CSS group ($p = 0.17$). Error bars represent ± SEM, and n values are displayed on the bars.

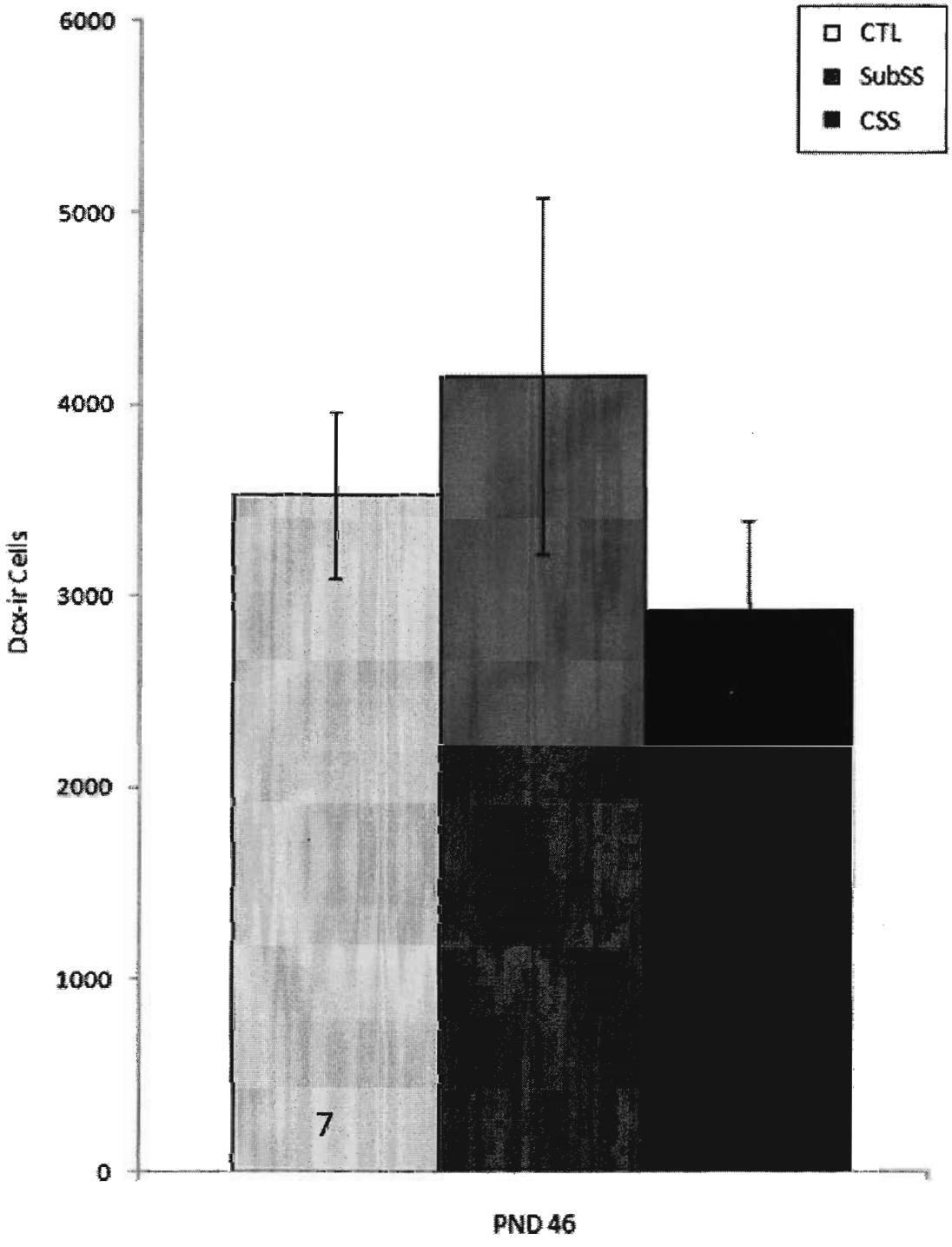

Figure 14. Experiment 1a. Mean number of Dcx-ir cells in the DG of the hippocampus on PND 46. No significant difference between the groups was found ($p = 0.41$). Error bars represent ± SEM, and n values are displayed on the bars

Experiment 1b – Investigating the effects of CSS on hippocampal neurogenesis generated after termination of the stressor, using BrdU and Dcx

BrdU: To examine the effects of stress on the cells generated after the stress procedure, rats were injected with BrdU on PND46 – 48. Age (PND 49, PND 75) X Stress Group ANOVA found that stress significantly increased the number of BrdU-ir cells when collapsed across age ($F_{1,20} = 4.60$, $p = 0.05$). The effect of age wasn't significant ($F_{1,20} = 1.22$, $p = 0.28$), and the interaction was not significant ($F_{1,20} = 0.06$, $p = 0.45$; see Figure 15).

Dcx: There was no significant difference among the groups in Dcx-ir cell numbers at PND 75 ($t_{14} = 0.87$ $p = 0.40$; see Figure 16).

Weight Data for Experiment 1

The CSS, SubSS and CTL did not differ in weight at PND 30 or at PND 45 ($F_{2,53} = 0.82$, $p = 0.45$ and $F_{2,53} = 1.15$, $p = 0.33$). Because the expected reduction in weight in CSS was not significantly different from CTL (see Figure 17), I explored the possibility that injection of BrdU on PND 30 – 32 minimized the group differences. Two-factor ANOVA of percent increase in body weight from PND 30 to 45 in CTL and CSS further grouped into injected and non-injected indicated that the percent increase in weight was lower in CSS than in CTL groups ($F_{1,44} = 9.64$, $p = 0.003$), and injected rats weighed less than non-injected rats ($F_{1,44} = 16.24$, $p < 0.0001$).

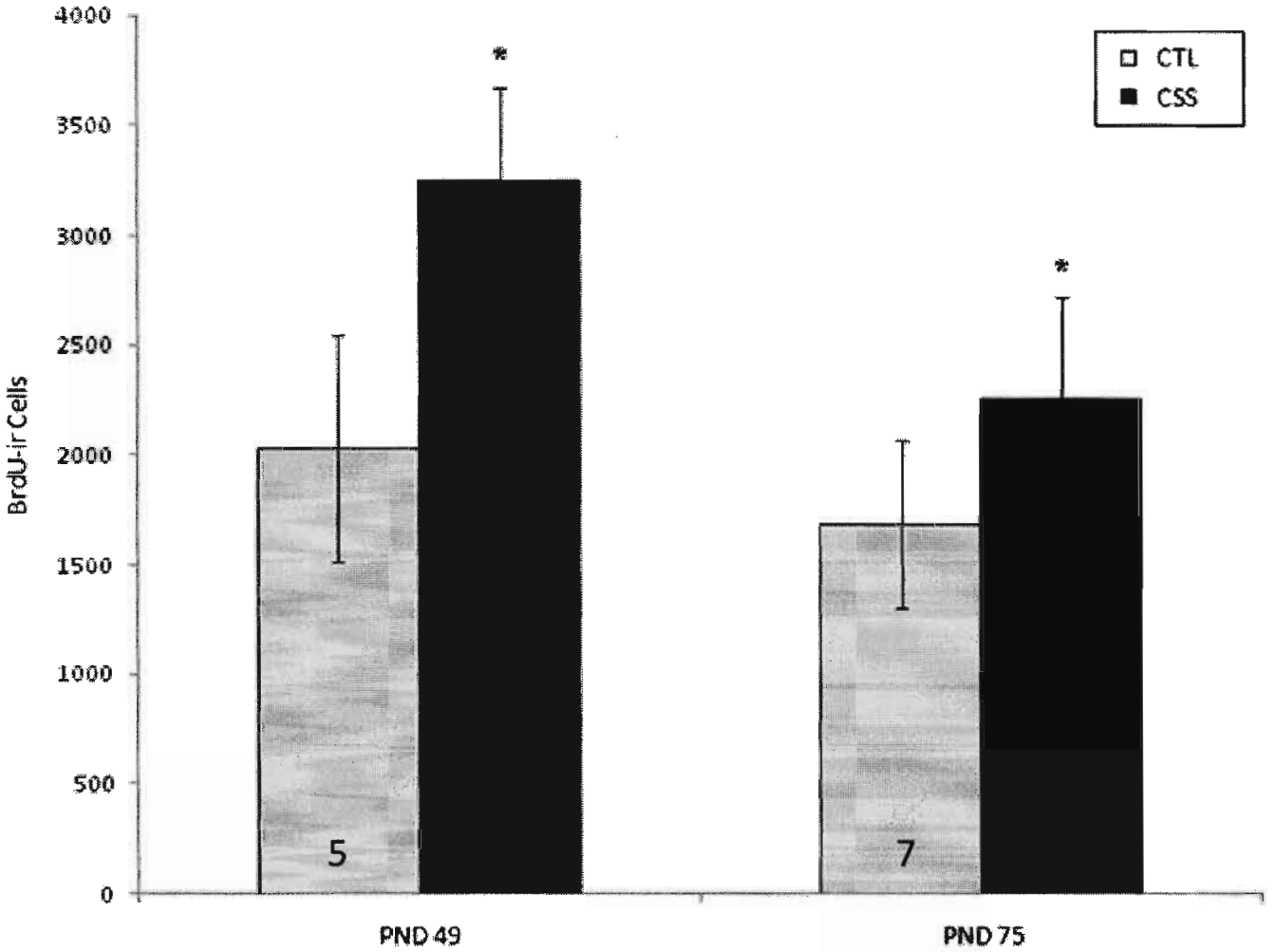

Figure 15. Experiment 1b. Mean number of BrdU-ir cells in the DG of the hippocampus on PND 49 and PND 75. Collapsed across age CSS had more BrdU-ir cells than CTL (*p = 0.05). Error bars represent ± SEM, and n values are displayed on the bars.

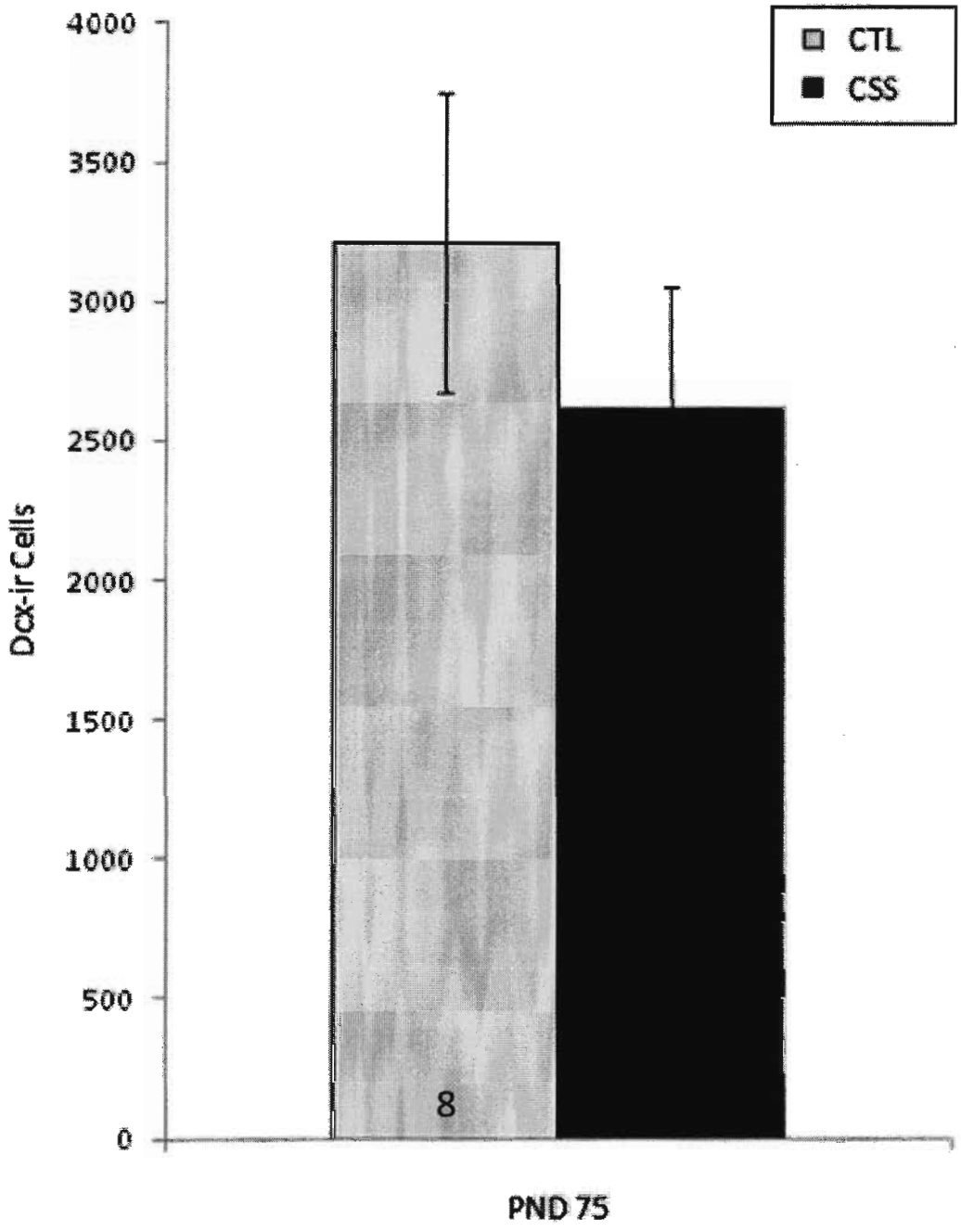

Figure 16. Experiment 1b. Mean number of Dcx-ir cells in the DG of the hippocampus on PND 75. No significant difference CTL and CSS was found ($p = 0.40$). Error bars represent ± SEM, and n values are displayed on the bars.

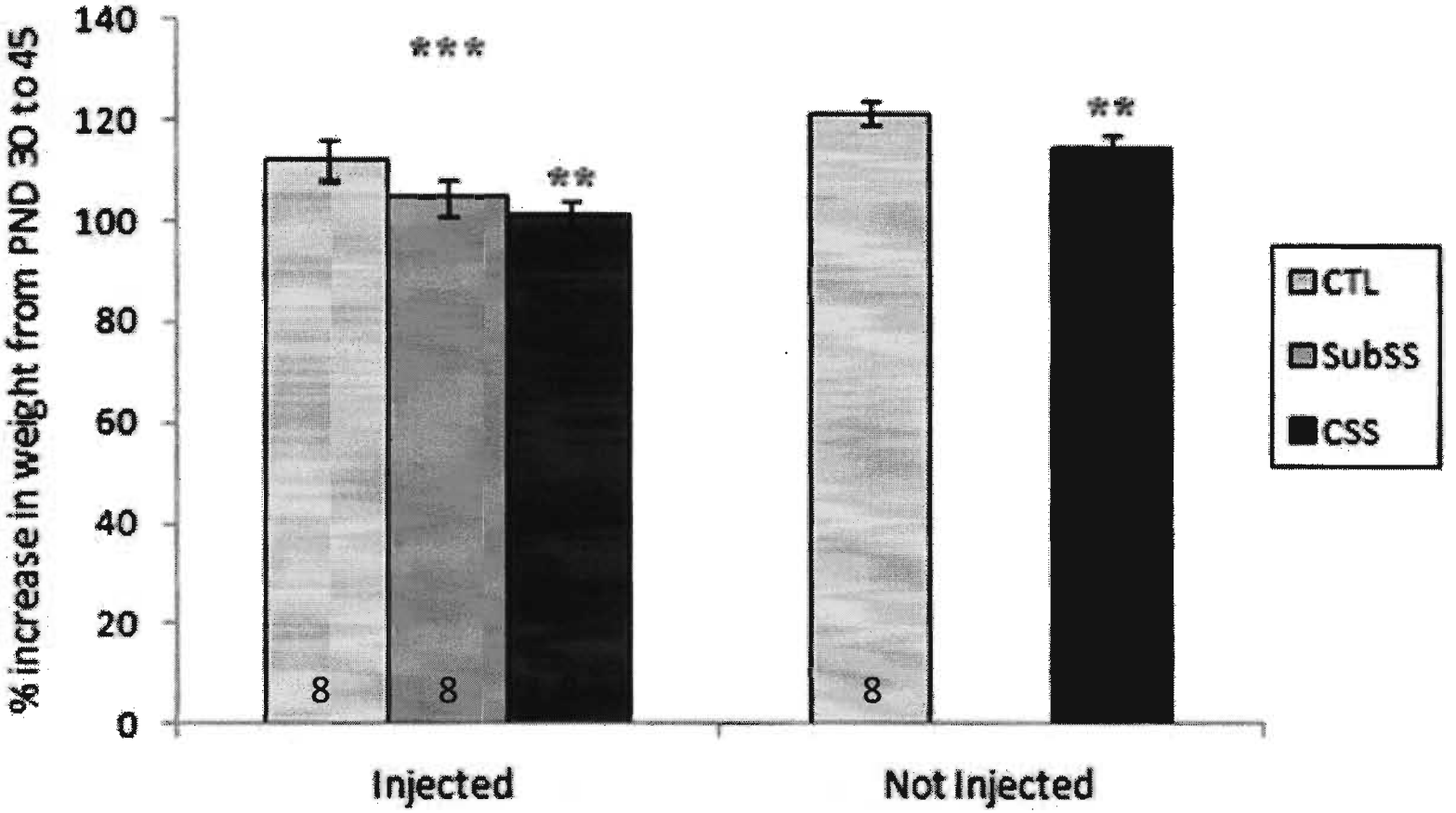

Figure 17. Experiment 1: Percent weight increase from PND 30 to 45. Rats were weighed at the beginning of the stress procedure (PND 30) and after the stressor was completed (PND 45). Above is the percent increase in weight from PND 30 to 45. Percent increase in weight was lower in CSS rats than CTL (*p = 0.003), and injected animals gained less weight than non-injected rats (***p < 0.0001). Error bars represent ± SEM, and n values are displayed on the bars.

Experiment 2: Endogenous Labels

Experiment 2a: Investigating the immediate and long-lasting effects of SubSS and CSS on hippocampal neurogenesis and the immune system, using Ki67, Dcx, and OX-42

Ki67: Each age group was analyzed separately because of large age differences. At PND 33, more Ki67-ir cells were found in the dorsal than the ventral region ($F_{1,12} = 5.16$, $p = 0.04$), and CSS rats had more Ki67-ir cells than CTL ($F_{1,12} = 4.77$, $p = 0.05$). At PND 46, more Ki67 cells were found in the dorsal DG region than in the ventral region ($F_{1,14} = 10.22$, $p = 0.06$), but the stress group effect was not significant ($F_{1,14} = 0.42$, $p = 0.53$). Similarly, at PND 75, there were still more dorsal Ki67 cells than ventral ($F_{1,12} = 21.63$, $p = 0.001$), but no significant effect of stress was seen ($F_{1,12} = 2.24$, $p = 0.16$; see Figure 18).

Dcx: Post hoc analysis for a Stress Group X Age interaction found that at both PND 46 and PND 75, there were more Dcx-ir cells in CSS compared to CTL rats ($t_{14} = 3.10$, $p = 0.008$, $t_{14} = 3.13$, $p = 0.007$). For both CTL and CSS, there were more Dcx-ir cells at PND 46 than at PND 75 ($t_{14} = 3.05$, $p = 0.009$, $t_{14} = 6.17$, $p < 0.0001$; see Figure 19).

OX-42: Mixed Factor (DG Region X Stress Group X Age) ANOVA for OX-42-ir cells found a DG Region X Age interaction ($F_{1,37} = 4.24$, $p = 0.02$). Post hoc for testing simple effects in the dorsal region indicated that there were more OX-42-ir cells at PND 33 than at PND 46 ($t_{25} = 3.26$, $p = 0.003$), and fewer at PND 46 than at PND 75 ($t_{27} = 4.06$, $p < 0.0001$). No difference was found between PND 33 and PND 75 ($t_{28} = 0.20$, $p = 0.85$) in the number of OX-42-ir cells in the dorsal region. In the ventral region, no difference was

found in OX-42-ir cells at PND 33 compared to PND 46 ($p = 0.28$), but there were more cells at PND 75 than at either PND 33 or PND 46 ($t_{28} = 3.51$, $p = 0.002$, $t_{27} = 2.50$, $p = 0.02$). There was no significant effect of stress on total number of microglia at any age (see Figure 20).

Mixed Factor (DG Region X Stress Group X Age) ANOVA for activated OX-42-ir cells revealed a DG Region X Age interaction ($F_{2,37} = 7.50$, $p = 0.002$). Post hoc testing for simple effects showed that in the dorsal region, the number of activated OX-42-ir cells was higher on PND 33 than on either PND 46 or PND 75 ($t_{25} = 4.65$, $p < 0.0001$, $t_{28} = 5.15$, $p < 0.0001$), but the number of cells did not differ between PND 46 and PND 75 ($t_{27} = 0.13$, $p = 0.90$). In the ventral region, the number of activated OX-42-ir cells on PND 33 did not differ from PND 46 or PND 75, ($t_{25} = 0.12$, $p = 0.91$, $t_{28} = 1.81$, $p = 0.08$). However there were more cells on PND 46 than PND 75 ($t_{27} = 2.00$, $p = 0.05$). When each age group was considered separately, CTL rats had more activated OX-42-ir cells than CSS rats only on PND 46 ($F_{1,11} = 7.50$, $p = 0.02$; see Figure 21).

Mixed Factor (DG Region X Stress Group X Age) ANOVA for percentage of activated OX-42-ir cells found a Stress Group X Age interaction ($F_{2,35} = 5.74$, $p = 0.007$). Post hoc analysis of Stress Group X Age interaction found no difference between CTL and CSS at PND 33 ($t_{12} = 0.66$, $p = 0.53$), or at PND 75 ($t_{14} = 0.94$, $p = 0.37$). However there were more activated OX-42-ir cells in CTL than CSS on PND 46 ($t_{11} = 3.87$, $p = 0.003$; see Figure 21).

Experiment 2b: Investigating if behavioural testing after CSS impacts any long-lasting effects of stress on hippocampal neurogenesis and the immune system, using Ki67, Dcx, and OX-42

Ki67: For adults (PND 74, PND 75) a Group X Testing (behaviourally tested or not) X Dentate Region (Dorsal,Ventral) ANOVA indicated that there were more Ki67-ir cells in the dorsal than in the ventral region ($F_{1,28} = 71.03$, $p < 0.0001$), and no other factor or interaction was significant (see Figure 18).

Dcx: Group X Testing (behaviourally tested or not) X Dentate Region (Dorsal,Ventral) ANOVA indicated there were more DCX-ir cells in CSS than in CTL rats ($F_{1,24} = 12.41$, $p = 0.002$). Post hoc analysis of the interaction of Group and Dentate Region ($F_{1,24} = 6.82$, $p = 0.015$) indicated that DCX-ir cell counts were higher in the dorsal than in the ventral dentate gyrus for CSS rats ($p = 0.005$), and the difference was not significant for CTL rats ($p = 0.49$; see Figure 19).

OX-42: For adults, (PND 74,PND 75), Group X Testing (tested or not) X Dentate Region (Dorsal,Ventral) ANOVA indicated there were more OX42-ir cells in dorsal than ventral region than in CTL rats ($F_{1,28} = 39.066$, $p < 0.0001$), and fewer OX42-ir cells in rats that underwent behavioural testing than those that did not ($F_{1,28} = 9.23$, $p = 0.005$). For activated OX42-ir cell counts, only the higher counts in the dorsal than ventral region were significant ($F_{1,28} = 5.32$, $p = 0.03$; see Figures 20 and 21).

Weight Data for Experiment 2

There was a significant Age X Group interaction ($F_{1,34}$ = 9.018.87, p = 0.005). Post hoc analysis of Age X Group interaction found that CSS rats did not differ from CTL at PND 30 (t_{18} = 0.83, p = 0.41), but CTL weighed more than CSS at PND 45 (t_{18} = 2.84, p = 0.01; see Figure 22).

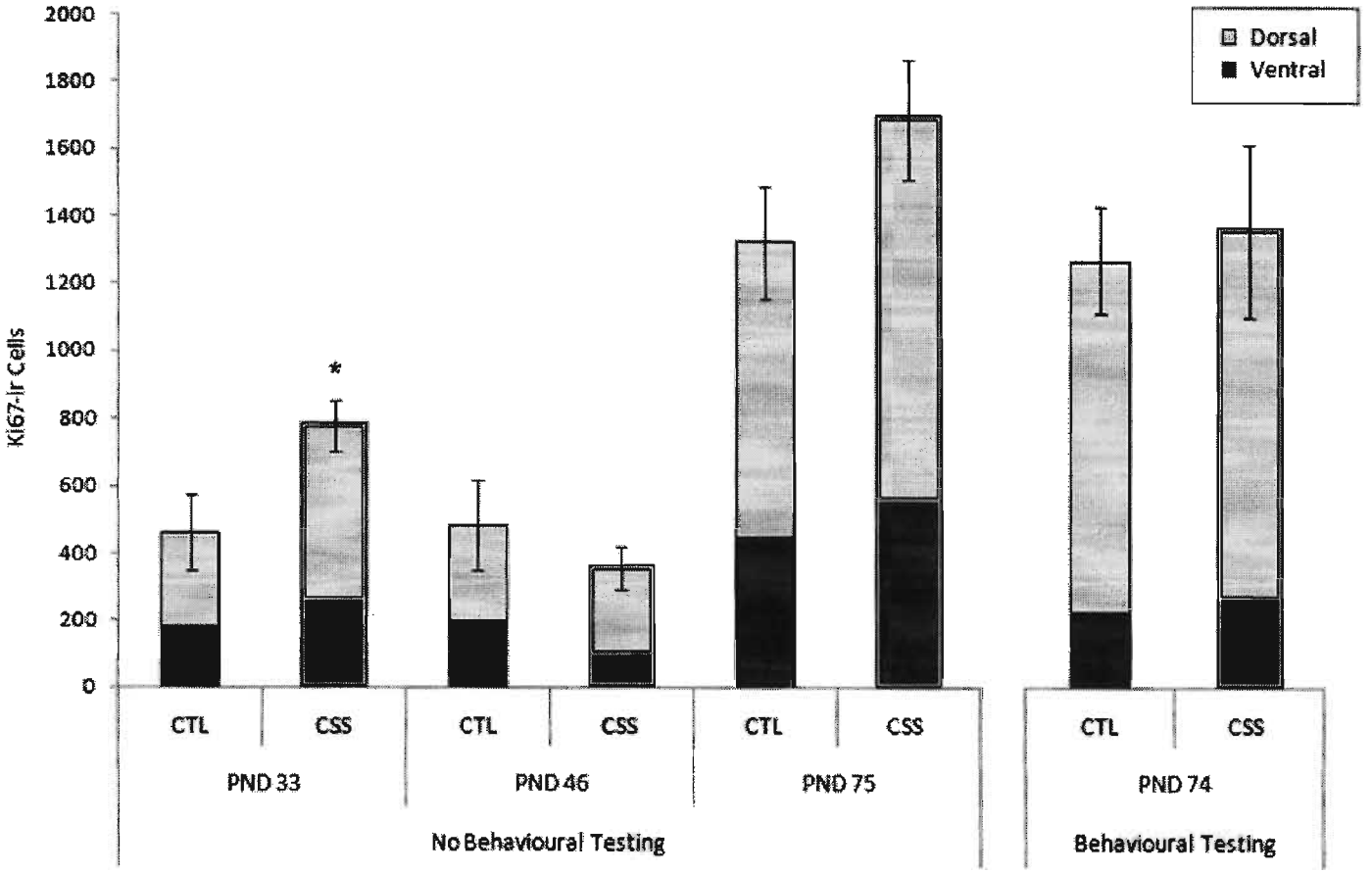

Figure 18. Experiment 2a (no behavioural testing) and 2b (behavioural testing): Ki67-ir cells in the DG of the hippocampus on PND 33, 46, and 75. Cell proliferation was increased at PND 33 as a result of stress ($p = 0.05$). There was also a trend that proliferation in the CSS rats remained high compared to CTL on PND 75. Error bars represent ± SEM, and n values are displayed on the bars.

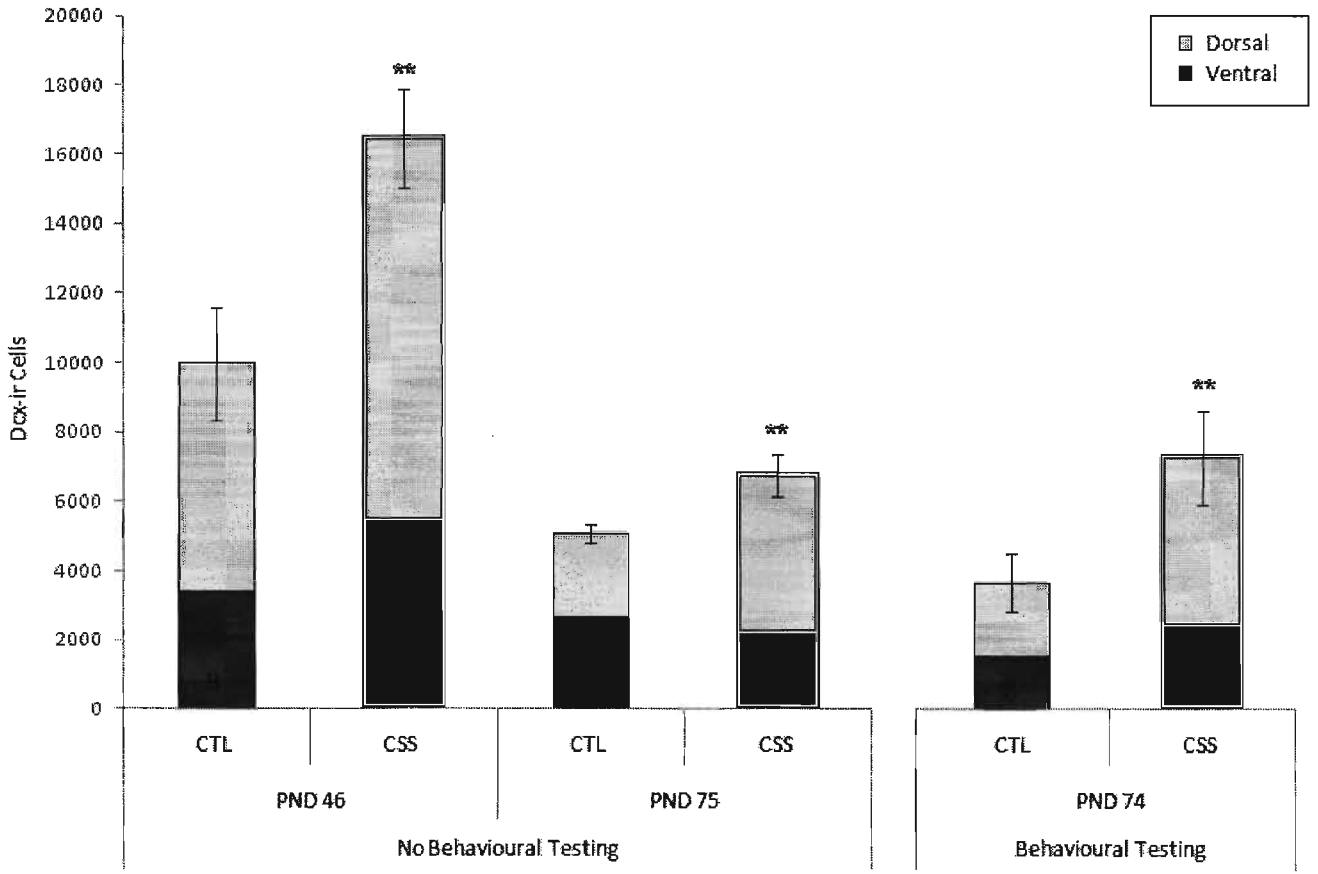

Figure 19. Experiment 2a (no behavioural testing) and 2b (behavioural testing): Dcx-ir cells in the DG of the hippocampus on PND 46 and 75. Conpared to CTL rats, CSS rats had significantly more neurogenesis at PND 46 (**p = 0.008), PND 75 (**p = 0.007), and PND 74 (**p = 0.002). Error bars represent ± SEM, and n values are displayed on the bars.

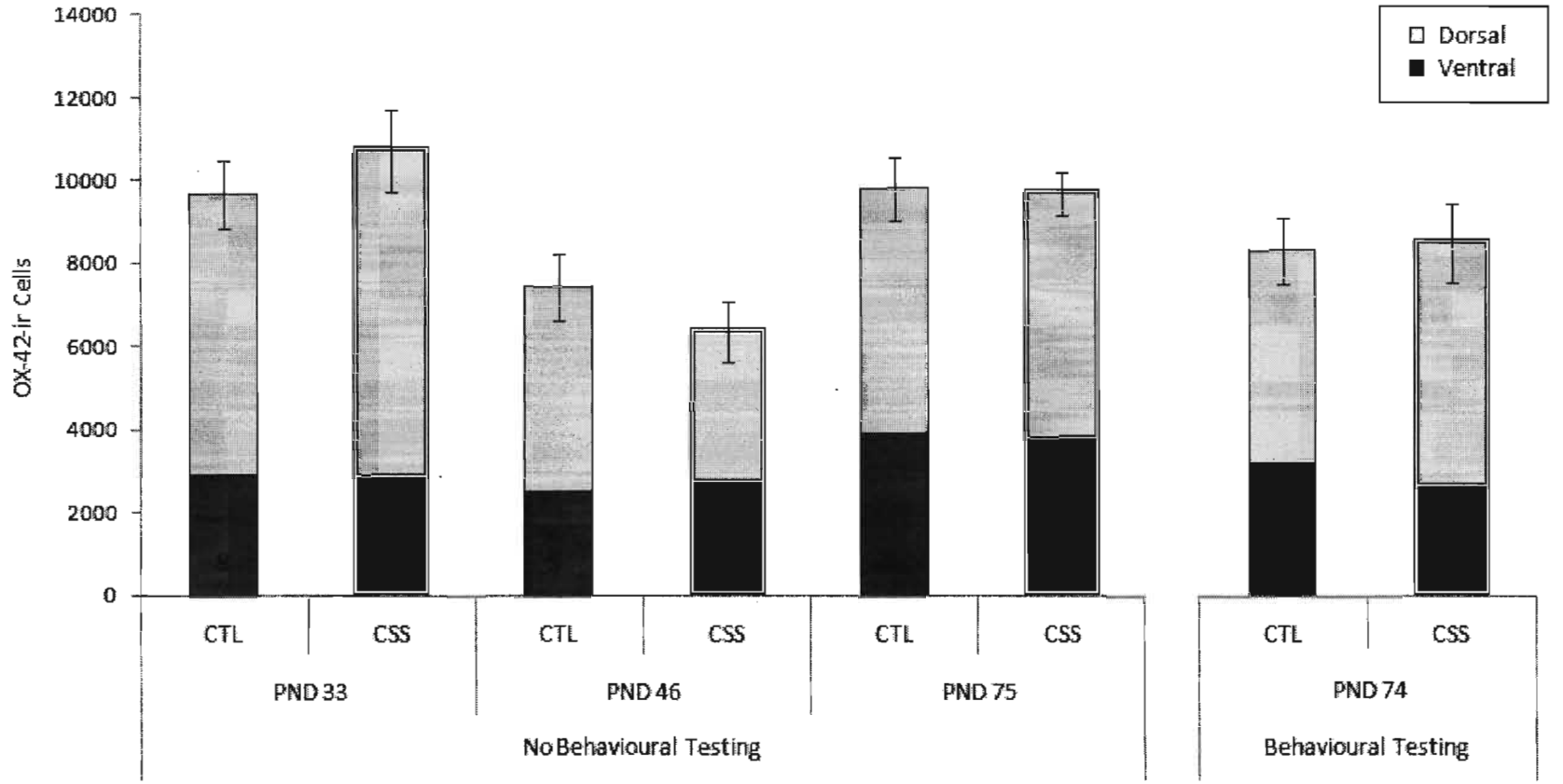

Figure 20. Experiment 2a (no behavioural testing) and 2b (behavioural testing): Total number of microglia detected by OX-42 in the DG of the hippocampus on PND 33, 46, and 75. There was no effect of group at any age. Error bars represent ± SEM, and n values are displayed on the bars.

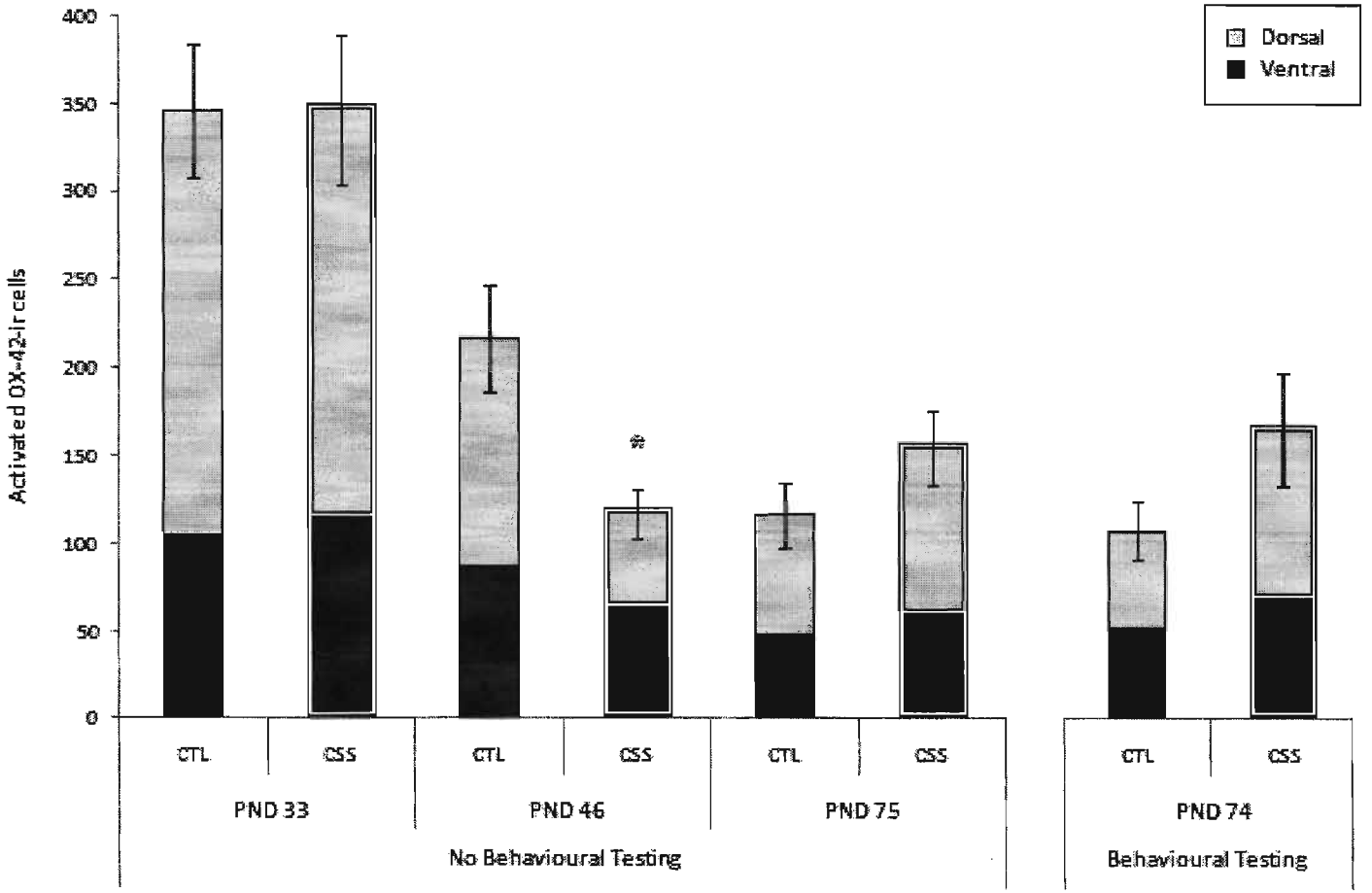

Figure 21. Experiment 2a (no behavioural testing) and 2b (behavioural testing): The number of activated microglia in the DG of the hippocampus marked with OX-42 on PND 33, 46, and 75. CTL and CSS groups did not differ at PND 33, 75 or 74, but at PND 46 CSS rats has fewer activated microglia compared to CTL (*p = 0.02). Error bars represent ± SEM, and n values are displayed on the bars.

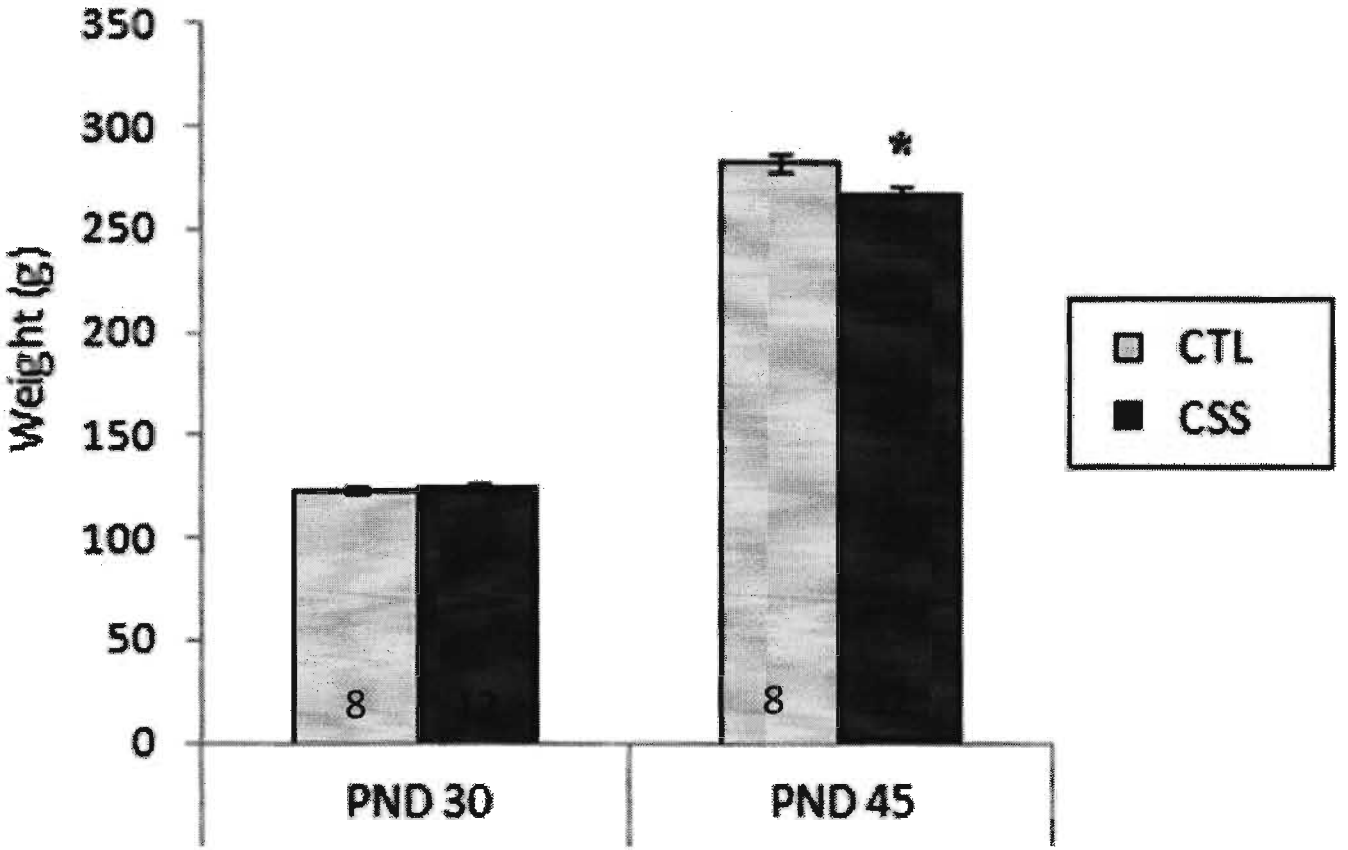

Figure 22. Experiment 2: Weights of rats on PND 30 and 45. Rats were weight at the beginning of the stress procedure (PND 30) and after the stressor was completed (PND 45). Whereas CTL did not differ from CSS at PND 30 ($p = 0.41$), CTL weighed more than CSS on PND 45 (*$p = 0.01$). Error bars represent ± SEM, and n values are displayed on the bars.

Discussion

Experiment 1 – BrdU Injections

Experiment 1a: Neither subacute nor chronic stress during adolescence had an effect on cell proliferation or survival, as demonstrated by BrdU- and Dcx-ir cells

In experiment 1a, there was no difference between the groups in BrdU- or Dcx-ir cells in the DG at either PND 33 or 46 (see Figures 13 and 14). Whether rats were stressed subacutely (SubSS, 3 days), chronically (CSS, 16 days), or not at all made no difference to the rate of neurogenesis during adolescence. As reflected by BrdU, stress did not affect the number of cells born at the beginning of the stress procedure (on PND 30 – 32), nor did it affect the survival of neurons generated throughout the stress procedure, as reflected by Dcx-immunohistochemistry.

There are many examples of both acute and chronic stress decreasing neurogenesis in adulthood (e.g. Czeh et al., 2001; 2007; Heine et al., 2004; Lee et al., 2006). However, there are a few examples that show that acute and shorter duration chronic stressors were not sufficient to affect neurogenesis, and the current finding that subacute stress had no effect on cell proliferation or survival coincides with the following results. For instance, a single exposure to predator odour was not enough to affect cell proliferation (Thomas et al., 2006), nor was 24 hours of sleep deprivation (Mirescu et al., 2006). Similarly, shorter-term chronic stressors, such as seven (Shors et al., 2006) or eight (Westenbroek et al., 2004) days of daily footshock did not affect neurogenesis. This is the first time our laboratory's model of adolescent stress has been used subacutely, and like the examples provided above, it may not be adequate to

evoke an appreciable stress response. However, the stress model has been applied chronically many times and has successfully evoked increased CORT levels and decreased weight gain across adolescence (e.g. McCormick et al., 2007; 2008; 2010), and in the current study, stressed rats gained less weight than controls (see Figure 17). Accordingly, that chronic stress lasting 16 days also had no effect on neurogenesis contrasts with the well-established finding that chronic stress during adulthood decreases neurogenesis. It is also in contrast to our previous finding that adolescent female Long Evans rats showed decreased neurogenesis in response to our stressor.

Experiment 1b: Chronic stress increased cell proliferation after termination of the stress procedure, as demonstrated by BrdU-ir cells, but not survival of neurons born throughout the stress procedure, reflected by Dcx-ir cells.

The results of experiment 1b demonstrate that chronic stress during adolescence elicited a long-lasting effect on cell proliferation, as evidenced by increased BrdU-ir cells in the CSS rats compared to CTL on both PND 49 and 75. However, survival of neurons generated throughout the stress procedure was not supported, as there was no difference in Dcx-ir cells on PND 75 between CSS and CTL rats. Upon closer inspection, it was noticed that not only did CSS rats weigh less than CTL rats, injected rats also weighed less than non-injected rats (Figure 17). Therefore, BrdU toxicity may have caused rats to experience weight loss, a possible side effect (Cooper-Kuhn & Kuhn, 2002), and possibly cytotoxicity.

BrdU can cause a number of developmental problems in the early postnatal rat, such as impaired lung (Nagai et al., 1993) and tooth development, reduced body weight,

and brain abnormalities (Kolb et al., 1999). In high doses in the adult rat, BrdU can be mutagenic, alter DNA stability (see reviews by Morris, 1991; Taupin, 2007), trigger apoptosis, and lengthen the cell cycle (Bannigan & Langman, 1979). However for a number of reasons, BrdU toxicity is not likely to be the underlying factor behind the observed weight loss in Experiments 1a and b. In adult rats, doses as high as 600 mg/kg have been demonstrated to mark cell genesis without any of adverse effects listed above (Cameron & McKay, 2001). The dose of BrdU used in the current study, 300 mg/kg, has been demonstrated to mark all new cell generation in the adult rat (Cameron & McKay, 2001) and adolescent mouse (He & Crews, 2007) without any reported adverse effects. Even when considering cumulative doses, Lie et al. (2002) exceeded that of this study, with a total cumulative dose of 2000 mg/kg (one injection of 200 mg/kg daily for ten days) administered to adult Sprague-Dawley rats with no reported side effects. It is also not likely that the strain of rat used in the current study, Long Evans, is more susceptible to the toxic effects of BrdU, as others (Epp & Galea et al., 2009) used a moderately high dose of BrdU (200 mg/kg) to mark neurogenesis in adult Long Evans rats with no reported adverse side effects.

It could be argued that because the brain is still maturing during adolescence, the adolescent rat may be vulnerable to the toxic effects of BrdU, much like neonatal rats, in which doses in excess of 50 mg/kg in neonatal rats has been found to be toxic (reviewed in Taupin, 2007). However, this is also not likely, as the neonatal animal is particularly vulnerable because the blood-brain barrier (BBB), which restricts the amount of BrdU entering the brain, has not formed yet. The BBB forms around PND 10

(Cameron & McKay, 2001), and by this logic, the adolescent rat brain should be protected from overexposure to BrdU.

Therefore, it is thought that the injection process is inherently stressful to adolescent rats. Others have acknowledged the deleterious effects of injection stress on other variables (Kaneko et al., 2007; Boehm et al., 1982), including estrous cycle irregularity in adolescent female rats (Raap et al., 2000), and accordingly, it is possible that the injection stress constitutes an acute stressor. As discussed above, acute stressors can affect neurogenesis (e.g. Gould et al., 1998; Kim et al., 2005). To the author's knowledge, this is the first report of injection stress being harmful to neurogenesis. Therefore, to study the effects of stress on neurogenesis in adolescence, endogenous markers of cell proliferation and neurogenesis may be better suited. Alternatively, decreasing the stress of BrdU administration may be accomplished by limiting the number of BrdU injections, or by giving BrdU orally.

Experiment 2 – Endogenous Labels

Experiment 2a: Whereas stress during adolescence increased cell proliferation transiently as reflected by BrdU-ir cells, chronic stress increased the number of Dcx-ir cells in the long-term, and transiently decreased the number of activated OX-42-ir cells

In experiment 2a, I attempted to test my original hypothesis – that like in adults and adolescent females (McCormick et al., 2010) stress impairs neurogenesis, and that the effect is long-lasting – this time without the confound of injections (Figure 22). It was found that SubSS increased the proliferation of cells in the DG as reflected by an increase in the number of Ki67-ir cells on PND 33, but CSS did not have an appreciable

effect on cell genesis, as there was no statistically significant difference between CTL and CSS on PND 46, or in adulthood (PND 75; see Figure 18), although there was a trend for augmented cell proliferation in CSS rats compared to CTL in adulthood. Because Ki67 is a proliferative marker of all cell genesis, in the current experiment, we cannot conclusively identify the Ki67-ir cells as neurons or glia, since progenitor cells within the DG have the capacity to differentiate into all neural cells, including oligodendrocytes, astroglia, and neurons (reviewed in Gage, 2000). That being said, it is very likely that most of the cells proliferating in the DG are in fact neurons. This observation is based on the work of various others, who demonstrated that the majority of proliferating cells in the dentate gyrus (upwards of 80%) do in fact differentiate into neurons (e.g. Cameron et al., 1993; van Praag et al., 1999; Barha et al., in press).

At all age groups (PND 33, 46, and 75), stress significantly increased the survival of immature neurons (Dcx-ir cells). Although there are very few studies investigating the effects of stress on the period of adolescence, the results of Experiment 2a are consistent with those of Toth et al., (2008), who also showed that in adolescent male rats, survival of newly generated neurons was amplified as a result of chronic stress during adolescence. Likewise, Barha et al. (in press) showed that chronic, intermittent restraint stress throughout adolescence resulted in a trend for both increased proliferation and survival of neurons compared to controls, observed in adulthood. Strikingly, the same stressor decreased neurogenesis in adolescent female rats. Although females were not studied in the current experiment, in agreement with the results of Barha et al. (in press), McCormick et al. (2010) previously showed that

adolescent stress decreases neurogenesis in female Long Evans rats. Therefore, it appears that adolescent male rats have some sort of compensatory mechanism for dealing with stress, but females are more vulnerable to the effects of stress, and sex hormones may mediate the effects of stress. The HPA axis is thought to be immature during adolescence, and it could be that stress during this time is handled differently between males and females, although the mechanism is not clear. The results reported here add to the growing body of literature demonstrating that stress can cause opposing effects on adolescent and adult animals and illustrate the problems with making comparisons across the two age groups, at least when neurogenesis is concerned.

It is presumed that the stressor I used would decrease neurogenesis if implemented during adulthood. This prediction is based on the vast literature that stress in adulthood decreases neurogenesis. Although the effects of this paradigm of stress during adulthood on neurogenesis have not been investigated, it has been implemented to compare the effects of stress administered during adolescence or adulthood on contextual and cued fear conditioning. Morrissey and colleagues (in press) had rats undergo a fear conditioning procedure the day after the last day of stress, or three weeks later. In fear conditioning, rats were exposed to aversive stimuli in the form of footshock, and learned to associate it with otherwise neutral stimuli: a tone (cued fear) or some aspect of the testing chamber (contextual fear). Whether rats learned to associate the neutral and aversive stimuli was measured by time spent immobile (freezing). Rats that were stressed in adolescence spent less time freezing

compared to controls during both context and cue testing, indicative of decreased memory, whereas the same stress procedure administered in adulthood had no effect on either contextual or cued fear conditioning memory. Similarly, Toth et al. (2008) used their model of chronic mild stress to show that stress implemented during adolescence or adulthood resulted in opposing effects: neurogenesis was increased as a result of adolescent stress in agreement with the present study, but when administered during adulthood, neurogenesis was depleted.

Another consideration is that in addition to our model of stress causing a stressful experience, it could also be providing an enriched environment. Environmental enrichment in adults has been found to positively affect neurogenesis (e.g. Nilsson et al., 1999; Olson et al., 2006), and it may be that while our stressor is in fact stressful to the adolescent rat, it may also provide a level of enrichment not considered before. The idea that stressors can also act as enrichment was originally put forth by Isgor et al. (2004), who postulated that although social crowding (one aspect of their variable social stressor) is stressful to the adult rat, it may be rewarding to the adolescent rat. Our stressor is a combination of social isolation and novel cage and cage partner. Whereas the isolation and novelty of the new cage and cage partner may in fact be stressful, any play behaviour that ensues could be rewarding to the adolescent rat (Douglas et al., 2004), despite the exposure to a stressful scenario and subsequent increase in CORT. Thus, it may be possible that our social stressor has aspects to it that are both beneficial and detrimental to neurogenesis, and the positive effects of enrichment are overriding the negative effects of stress. However, this interpretation is unlikely, as our model of

chronic stress has been found to impair spatial memory, regardless of effect on neurogenesis (discussed further below). It is also conceivable that HPA axis is simply capable of dealing with mild stressors like the one instituted in the current study, at least that there are yet unknown mechanisms in place that supersede the negative effects of CORT on neurogenesis. The fact that other stressors, such as exercise (van Praag et al., 1999; Eadie et al., 2005) and electroconvulsive shock therapy (Scott et al., 2000) can increase neurogenesis attests to this prediction.

Aside from the observed differences between CTL and CS rats, the overall level of neurogenesis decreased from adolescence to adulthood, as reflected by Dcx-immunohistochemistry (see Figure 19). These results uphold the work of those who have shown a linear, age-related decline in neurogenesis over the course of life (He & Crews, 2007; Heine et al., 2004; Kuhn et al., 1996; Leuner et al., 2007). However, the current finding that proliferation levels were higher in adult rats compared to adolescent rats (see Figure 18) is perplexing, as He and Crews (2007) clearly demonstrated that proliferation in adolescent mice was higher than their adult equivalents. The rate of neurogenesis is thought to peak two weeks after birth (Schlessinger et al., 1975) and then decline steadily, while other cell populations remain stable across life. It is possible that the discrepancy is a result of variability in the immunohistochemistry assay. Regardless, because of the large differences across age, statistical analysis was performed separately at each age group.

The fact that other cell populations remain stable throughout life was also demonstrated in this study, as the total microglia population was relatively constant

across adolescence and into early adulthood. Although there was a slight depression in total microglia population irrespective of condition on PND 46 (see Figure 20), there was no age-related decline in the number of microglia across the observed period of life.

It was found that stress affected the activation of microglia transiently, as on PND 46 the number of activated microglia was decreased in CSS rats compared to CTL. Thus, in the current study, chronic mild social stress decreased the number of activated microglia, possibly via anti-inflammatory cues, and was associated with increased neuron survival also on PND 46. These results are in contrast to those of Ekdahl et al. (2003) and Monje et al. (2003), who both showed that immune stress by LPS increased the production of activated microglia, and decreased neurogenesis. It could be that the source of stress is particularly important, and immune stressors such as LPS and psychosocial stressors have differential effects on the immune system. This theory is supported by the work of Butovsky et al. (2005), who demonstrated that in vitro, LPS caused microglia to become activated, and through inflammation, to be cytotoxic towards neurons. Strikingly, certain anti-inflammatory cytokines, such as IFN-γ and IL-4 were capable of activating microglia, but promoting differentiation of neurons. Hence it is possible that certain stressors are supportive of neuron survival through activation of microglia.

Considering all options, it is conceivable that our laboratory's model of mild social stress was not sufficient to elicit an immune response – perhaps environmental stimuli are not sufficient to induce an effect on microglia. Yet microglia reportedly respond to even the slightest assault (reviewed in Graeber, 2010). Moreover, others

have used physical stressors to show that microglia can be activated by environmental factors. For instance, Nair and Bonneau (2006) showed that four sessions of restraint stress-induced increase in CORT resulted in increased proliferation of microglia in adult male mice. Similarly, Sugama et al. (2007) demonstrated a pro-inflammatory effect of acute stress on microglial activation. Rats and mice were restrained and immersed in water (all but the head) for two hours, and euthanized immediately after. It was found that acute stress in both species encouraged resting microglia to become activated. It may be that our stressor simply does not constitute an immune challenge. Nonetheless, the observation that the number of activated microglia was reduced as a result of stress, indicating not that stress did not have any effect on the immune system, but that stress was detrimental to microglia function. There is evidence that corroborates our findings, as CORT has been found to decrease proliferation of activated microglia. Specifically, when the synthetic glucocorticoid, dexamethasone, was administered daily for four days, it decreased the proliferation of activated microglia cells in adult male Sprague-Dawley rats (Woods et al., 1999). It may also be that chronic stress and simultaneous CORT secretion is detrimental to all cells, not just newly generated neurons. This is plausible, as stress-induced CORT secretion is associated with increased glutamate release (Moghaddam et al., 1994) and possible glutamate excitotoxicity, especially in regions such as the hippocampus with high concentrations of GRs and MRs.

Thus, the involvement of stress in activation of microglia and the ensuing pro- or anti-neurogenic effect is complex. It may be that chronic stress decreases the number of activated microglia, subsequently decreasing the release of inflammatory cytokines,

and allowing for neurons to proliferate. Also worth mentioning is the observation that the number of activated microglia decreased in CTL rats in a stepwise fashion from PND 33 to 75 (see Figure 21). It is known that upon activation, microglia acquire motility (Nimmerjahn et al., 2005), and as neurogenesis decreases throughout life, the chemical cues associated with new neuron proliferation may also decrease, thus recruiting fewer activated microglia to the GCL layer of the DG.

Experiment 2b: The observed increase in neurogenesis as a result of chronic stress in adolescence is not affected by subsequent behavioural testing

In Experiment 2b, a group of rats underwent the stress procedure during adolescence, and were subjected to behavioural testing on PND 46 – 47 and again on PND 70 – 71. These rats were euthanized on PND 74, and immunohistochemistry was performed on brain sections to investigate if behavioural testing altered the stress-effect on neurogenesis. The results from these animals were compared to a group of rats in Experiment 2a, which were stressed the same and euthanized on PND 75, but did not experience behavioural testing. Regardless of behavioural testing, the rats all showed the same patterns of differences in all measures (see PND 75 and 74 in Figures 18 – 21). In adulthood, neither group showed differences between CTL and CSS conditions in Ki67, or OX-42 (total population or activated), whereas Dcx-ir was increased in both groups. Gould et al. (1999) found that learning tasks dependent on the hippocampus increased the number of BrdU-ir cells in the DG. In the current study, chronic stress increased survival of new neurons, but learning and memory training did

not further enhance the levels of neurogenesis. It appears that stress precluding behavioural testing nullifies any effect of behaviour on neurogenesis.

Although the functionality of neurogenesis has not been directly addressed here, the rats in the current study that were stressed and behaviourally tested showed impaired performance on a spatial location task (Appendix B). These results coincide with our previous finding that spatial location memory is also impaired in adolescent female rats of the same species (McCormick et al., 2010). This observation is made despite current and previous findings that our stressor decreases neurogenesis in adolescent female rats (McCormick et al., 2010), but has the opposite effect in their male equivalents (Appendix B). Thus, at least in adolescent rats, spatial location memory appears to not be solely tied to hippocampal neurogenesis, or at least there are more factors involved. Others have shown evidence to contradict the role hippocampal neurogenesis as being pivotal in the stress-induced decrease in acquisition of hippocampal-dependent tasks. Bartolomucci et al. (2002) showed enhanced performance on a hippocampal-dependent task was simultaneously associated with decreased neurogenesis, and Shors et al. (2002) showed decreased neurogenesis in response to stress, but no impairments on hippocampal-dependent tasks. Therefore, it appears that stress can affect both neurogenesis and the ability of an animal to consolidate hippocampal-dependent tasks. The relationship between neurogenesis and memory appears to at least be correlated, in that they both respond to environmental influences, but whether a causational relationship exists remains to be determined.

Also of note is the slight difference between the number of total microglia cells on PND 74 and 75. Although there was no effect of stress at either age, behavioural testing did seem to decrease the total number of microglia on PND 74 (although not significantly), regardless of group. It is possible that the learning and memory associated with the behavioural testing negatively affected microglia survival, but how that could occur is not clear. More research on the matter is necessary to elucidate if the observed effect is real, or by chance.

Summary

My original hypothesis was that as in adulthood, stress during adolescence would impair neurogenesis, and that the effect would be long-lasting. Here it is shown that contrary to my hypothesis, chronic mild social stress during adolescence increased both proliferation and survival of neurons in the male Long Evans rat. In agreement with part of my hypothesis, the observed augmentation of neuron survival was long-lasting. It appears that stress experienced during adolescence, a sensitive period of brain development, is rewiring the hippocampus, causing long-lasting changes. This postulate coincides with that of Tsoory et al. (2008), who suggested that stress experienced in adolescence may underlie adult-onset psychopathies. The observation that stress also transiently decreased the number of activated microglia in the DG was unexpected, and sheds light on the complexity of microglial activation.

These results are in contrast to the many studies that show that chronic stress in adulthood decreases neurogenesis, and further dissociate the notion that CORT always decreases neurogenesis. Thus, the effects of at least mild stress in adulthood and

adolescence are manifested in very different way, and this study demonstrates the limitations in attempting to make parallels between the two age groups. In addition to differences between adolescence and adulthood, the results of the current study and previous research (McCormick et al., 2010; Barha et al., in press) suggest that stress causes differential effects in male and female adolescent rats. That mild stress exposure in male and female adolescent rats has opposing effects of neurogenesis suggests that males handle stress differently than females, and could be the result of sex hormones mediating the stress response. However further research is necessary to understand the apparent sex difference in stress responsivity in adolescence.

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Appendix A - Materials

Table 1 – List of Materials

Product Name	Catalogue Number	Supplier
BrdU Injections		
5-Bromo-2'-deoxyuridine	B5002	Sigma
Sodium Chloride Solution	S8776	Sigma
Transcardial Perfusions		
Sodium Chloride	SOD002.205	Caledon
Sodium Phosphate Dibasic	SPD307.500	Caledon
Sodium Phosphate Monobasic	SX0710-1	EMD Chemicals
Paraformaldehyde	P6148-1KG	Sigma
Cryoprotectant		
Sodium Chloride	SOD002.205	Caledon
Sodium Phosphate Dibasic	SPD307.500	Caledon
Sodium Phosphate Monobasic	SX0710-1	EMD Chemicals
Polyvinylpyrrolidine	PVP10-500g	Sigma
Sucrose	8720-1	Caledon
Ethylene Glycol	1024466-4L	Sigma
Tissue Culture Plates (48-well)	07-200-86	Fisher
Immunohistochemistry		
Sodium Chloride	SOD002.205	Caledon
Sodium Phosphate Dibasic	SPD307.500	Caledon
Sodium Phosphate Monobasic	SX0710-1	EMD Chemicals
Triton X-100	TRX777.100	BioShop
Goat Serum	G9023	Sigma
Horse Serum	H0146	Sigma
Rabbit Serum	R9133	Sigma
Hydrogen Peroxide	4060-1	Caledon
Monoclonal Rat Anti-Bromodeoxyuridine	OBT0030	Accurate Chemicals & Scientific Corporation
Biotinylated Anti-Rat IgG	BA-4000	Vector
Doublecortin (C-18)	SC-806	Santa Cruz Biotechnology
Biotinylated Anti-Goat IgG	BA-9500	Vector
Ki67 Antigen Antibody (rabbit polyclonal)	VP-K451	Vector
Biotinylated Anti-Rabbit IgG	BA-1000	Vector
Mouse Anti-Rat CD11b	MCA275GA	AbD Serotec
ABC Kit Standard	PK-6100	Vector
ABC Kit Mouse IgG	PK-6102	Vector

VIP Peroxidase Substrate Kit	SK-4600	Vector
DAB Peroxidase Substrate Kit	SK-4100	Vector
Tissue Culture Plates (12-well)	07-200-81	Fisher
Glass Slides	12-550-15	Fisher
Cover Slips	12-545M	FIsher
Xylenes	98000-1	Caledon

Appendix B – Immunohistochemistry Protocol

Table 2 –Serum and Antibodies Reference Table

Protocol	Serum Origin	1° Ab			2° Ab		
		Origin	Name	Dilution	Origin	Name	Dilution
BrdU	Rabbit	Rat	Anti-BrdU	1:200	Rabbit	Anti-Rat	1:900
Dcx	Horse	Goat	Anti-Dcx	1:1000	Horse	Anti-Goat	1:500
Ki67	Goat	Rabbit	Anti-Ki67	1:5000	Goat	Anti-Rabbit	1:500
Microglia	Horse	Mouse	Anti-Rat CD11b	1:10 000	Horse	Anti-Mouse	1:750

1) Wash sections 3 times (5 minutes each) in 0.1 M PBS-X.

2) Pretreatment

BrdU: Denature DNA in 2 N HCl for 30 minutes in an oven at 37°C. Neutralize acid by washing sections 3 times (5 minutes each) in 0.1 M PBS.

Ki67: Incubate sections in 10 mM SSC for 30 minutes at 80°C. Keep sections in 10 mM SSC and allow sections to cool to room temperature (20 minutes). Wash sections 3 times (5 minutes each) in 0.1 M PBS-X.

3) Block sections once (for 30 minutes) in 0.3% H_2O_2.

4) Wash sections 3 times (5 minutes each) in 0.1 M PBS-X.

5) Incubate sections in blocking solution for 90 minutes.

6) Incubate sections in primary antibody overnight.

7) Wash sections 3 times (5 minutes each) in 0.1 M PBS-X.

8) Incubate sections in secondary antibody for 2 hours.

9) Wash sections 3 times (5 minutes each) in 0.1 M PBS-X.

10) Incubate sections with ABC Kit for 45 minutes.

11) Wash sections 3 times (5 minutes each) in 0.1 M PBS-X.

12) Incubate sections with DAB or VIP Kit for 2-6 minutes.

13) Wash sections 3 times (5 minutes each) in 0.1 M PBS-X.

14) Put sections in distilled water, and mount on slides.

Note: Sections were continuously agitated throughout the protocol. All steps were performed at room temperature unless specified otherwise.

Appendix C – Behavioural Testing Data

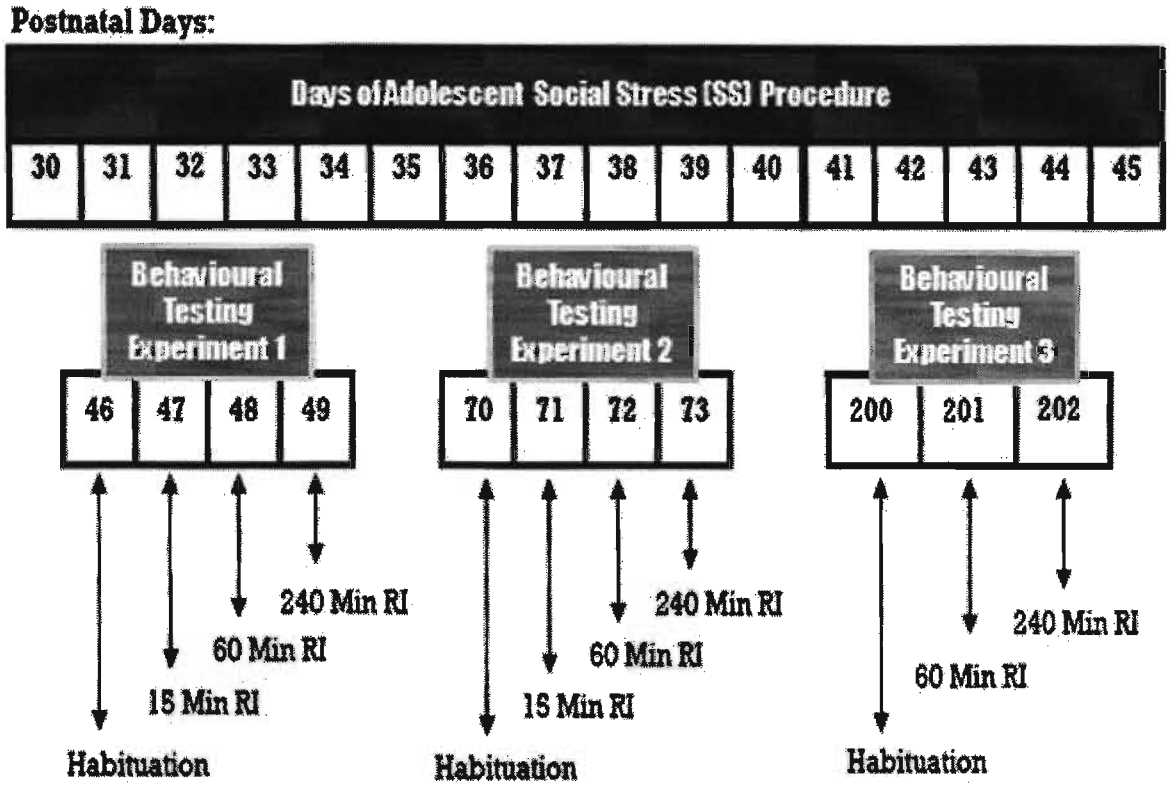
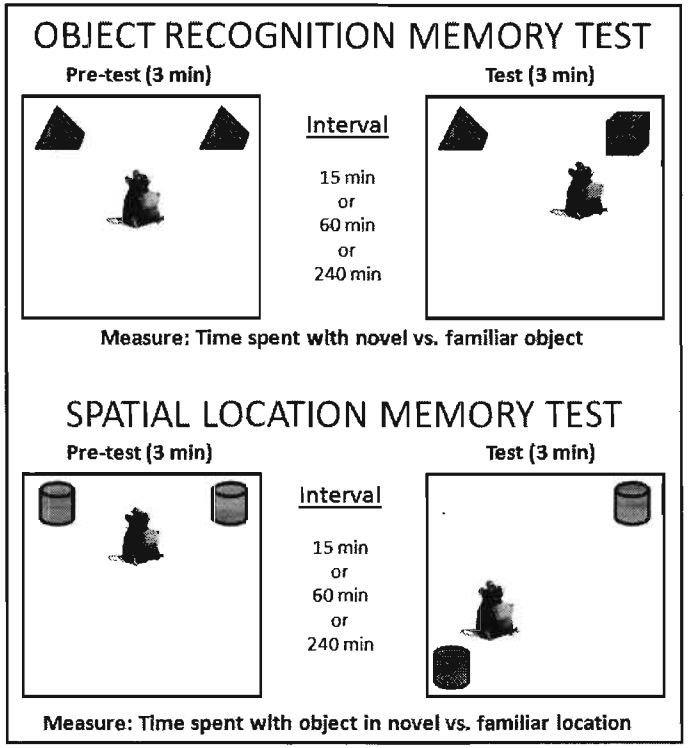


Figure 23. Experimental design for rats destined for behavioural testing. Rats were stressed from PND 30 to 45. On PND 46 – 49, and 70 – 73, rats underwent spatial location and object recognition training, and on PND 200 – 202, rats underwent spatial location testing only. On the first day of each session (PND 46, 70 and 200), rats were habituated to the testing arena for 30 minutes. On each subsequent day (PND 47 – 49, 71 – 73, and 201 – 202), rats were tested for object recognition and/or spatial location.

A)



B)

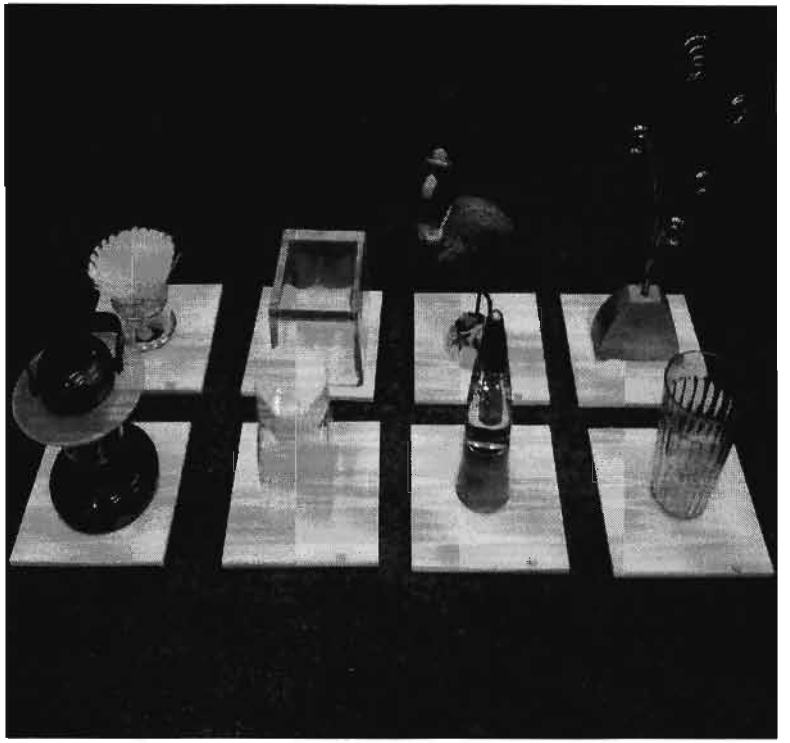


Figure 24. Rats were behaviourally tested to assess the intervening effects of learning and memory on the stress effect on neurogenesis. A) The testing arena set-up for object recognition and spatial location testing. For object recognition testing, after habituation the day before, rats were placed in the testing arena with two of the same objects for the pre-test phase, lasting three minutes in all cases. After a 15, 60, or 240 minute interval, rats were placed back in the arena, with one novel object, and one familiar object. Time spent with the novel versus familiar object was assessed. For spatial location testing, rats were placed with two new objects for the pre-test phase, and after the determined interval, were placed back in the arena with the same objects, one of them in a novel location. Time spent with the familiar object in the novel location was assessed. B) Objects used in testing arena.

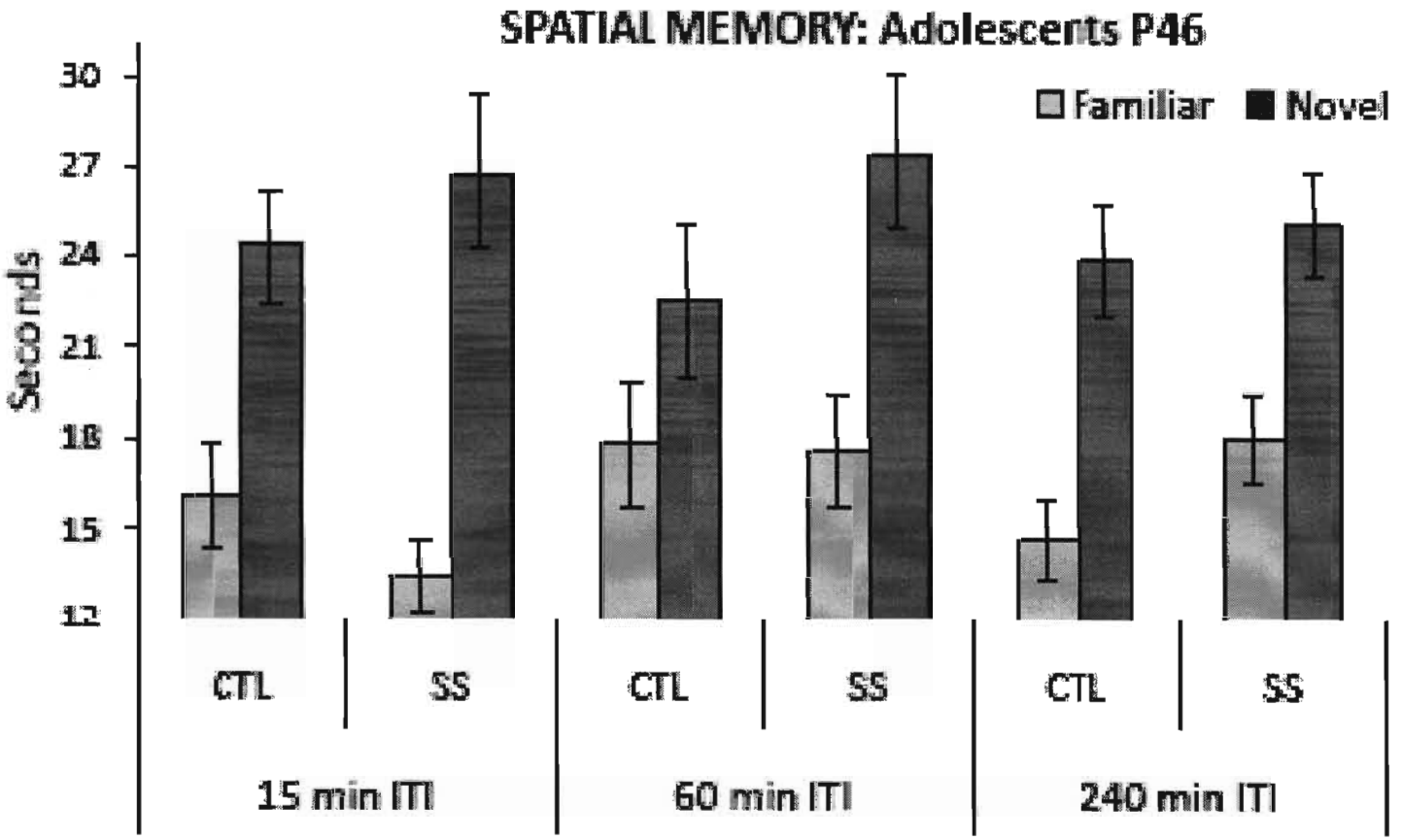


Figure 25. Experiment 1: Spatial location testing at PND 46 revealed that in male adolescent rats, time spent with the object in the novel location was not affected by social stress (SS), regardless of the length of inter-trial interval (ITI; 15, 60, or 240 minutes).

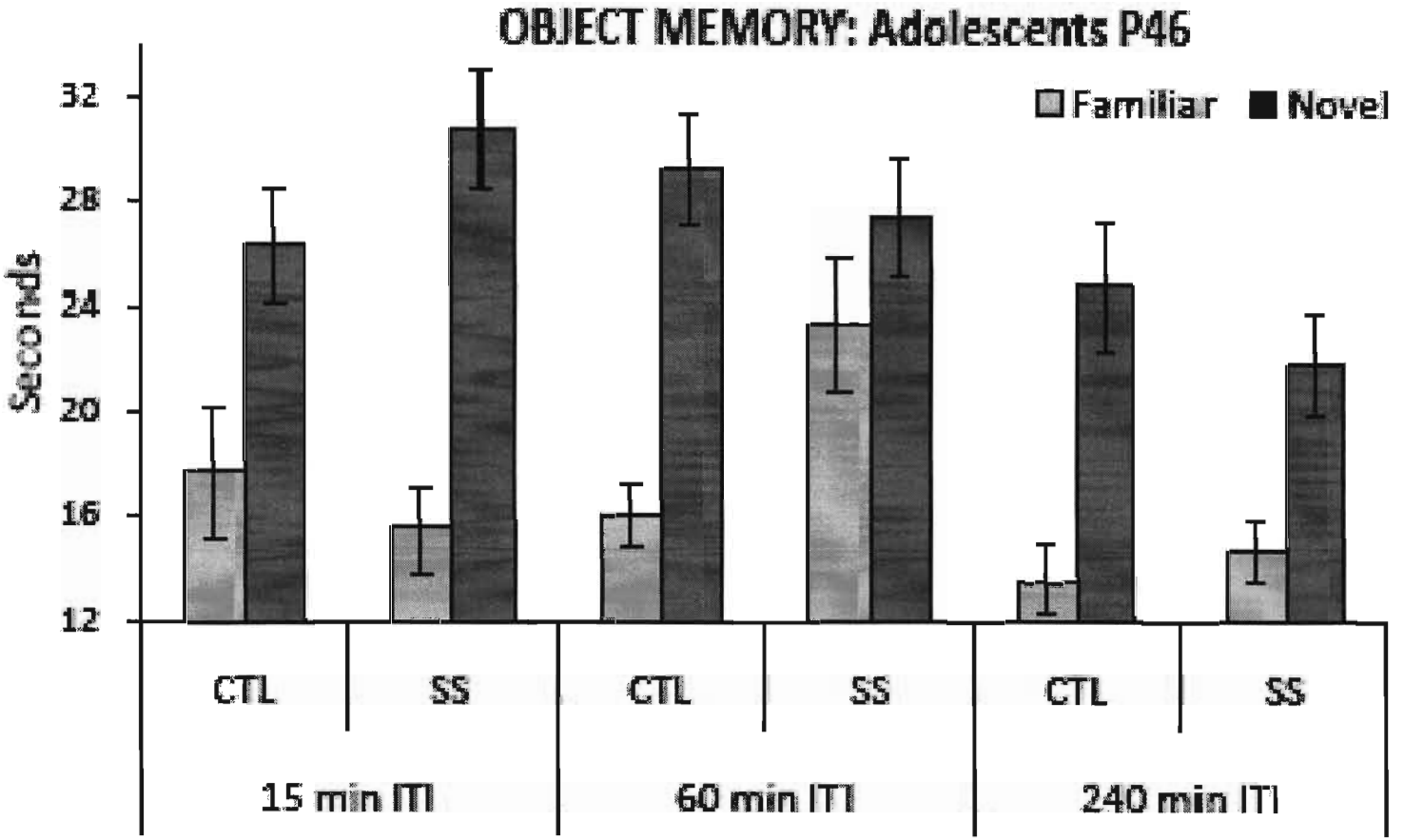


Figure 26. Experiment 1: Object recognition memory testing on PND 46 showed that compared to CTL rats, SS had no effect on time spent with the novel object irrespective of the length of ITI.

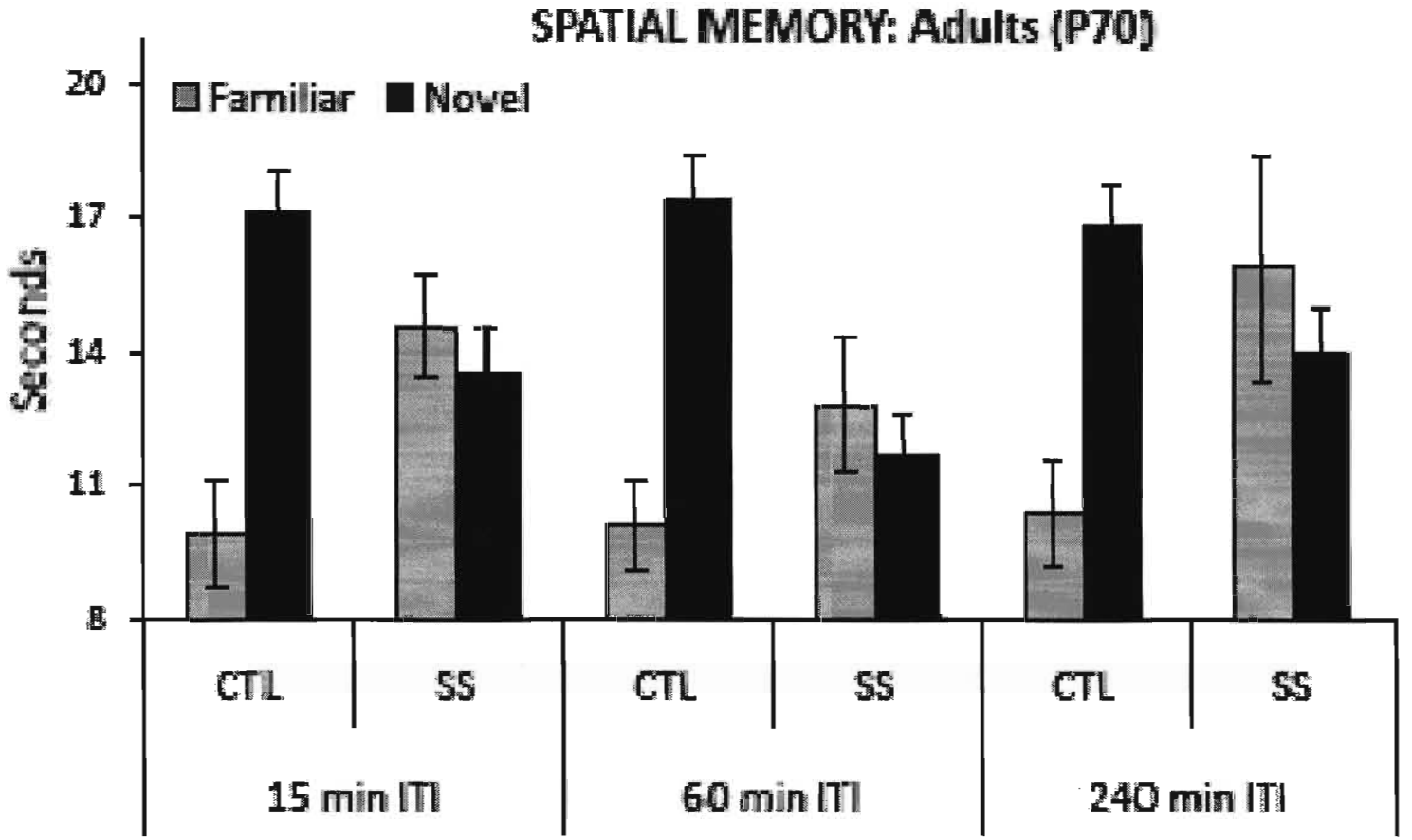


Figure 27. Experiment 2: When tested again on PND 70, spatial memory was hindered by SS, as there was no difference between time spent between the object in the familiar and novel locations, indicating memory deficits.

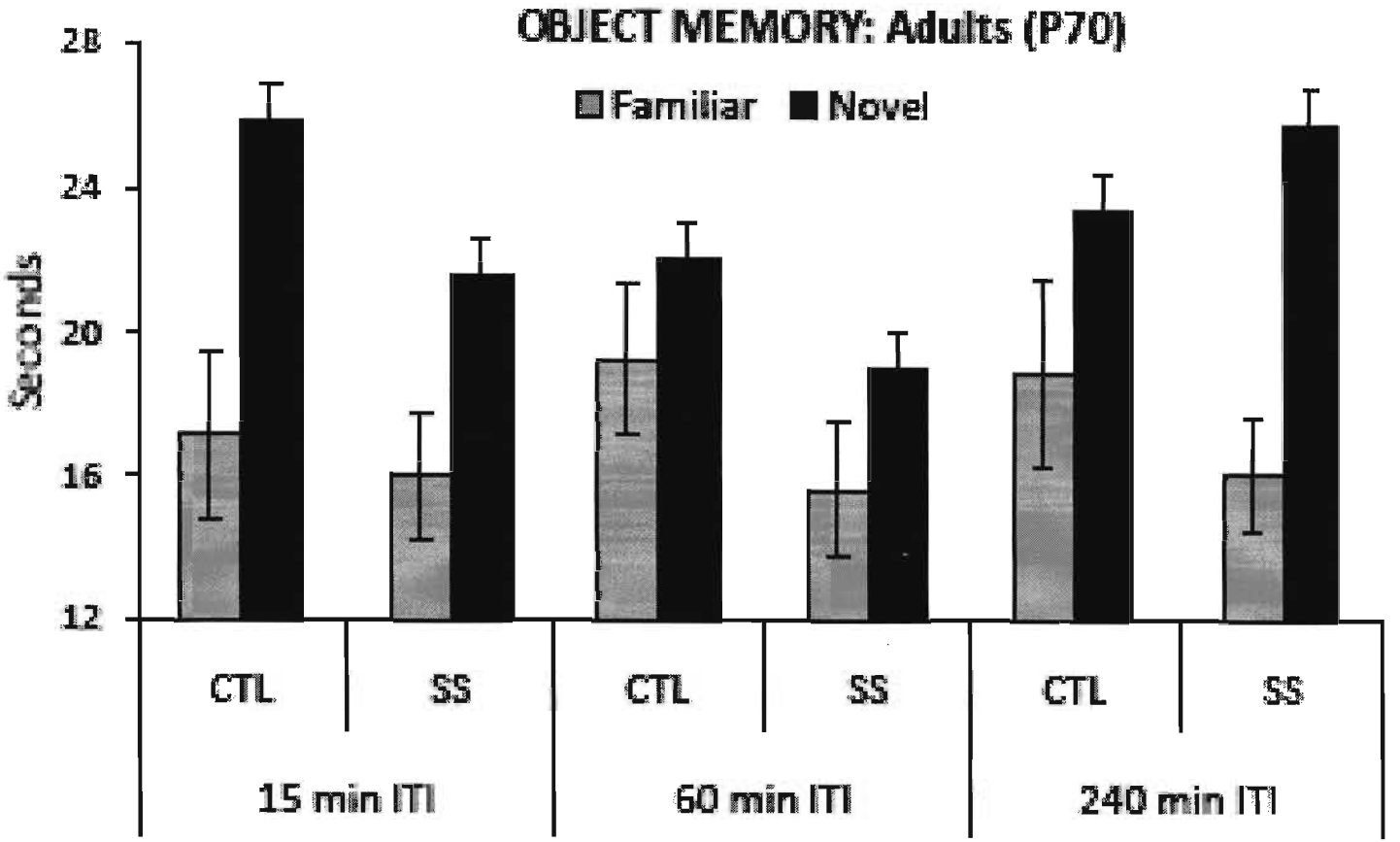


Figure 28. Experiment 2: Object recognition memory was not affect by SS when rats were tested again on PND 70.

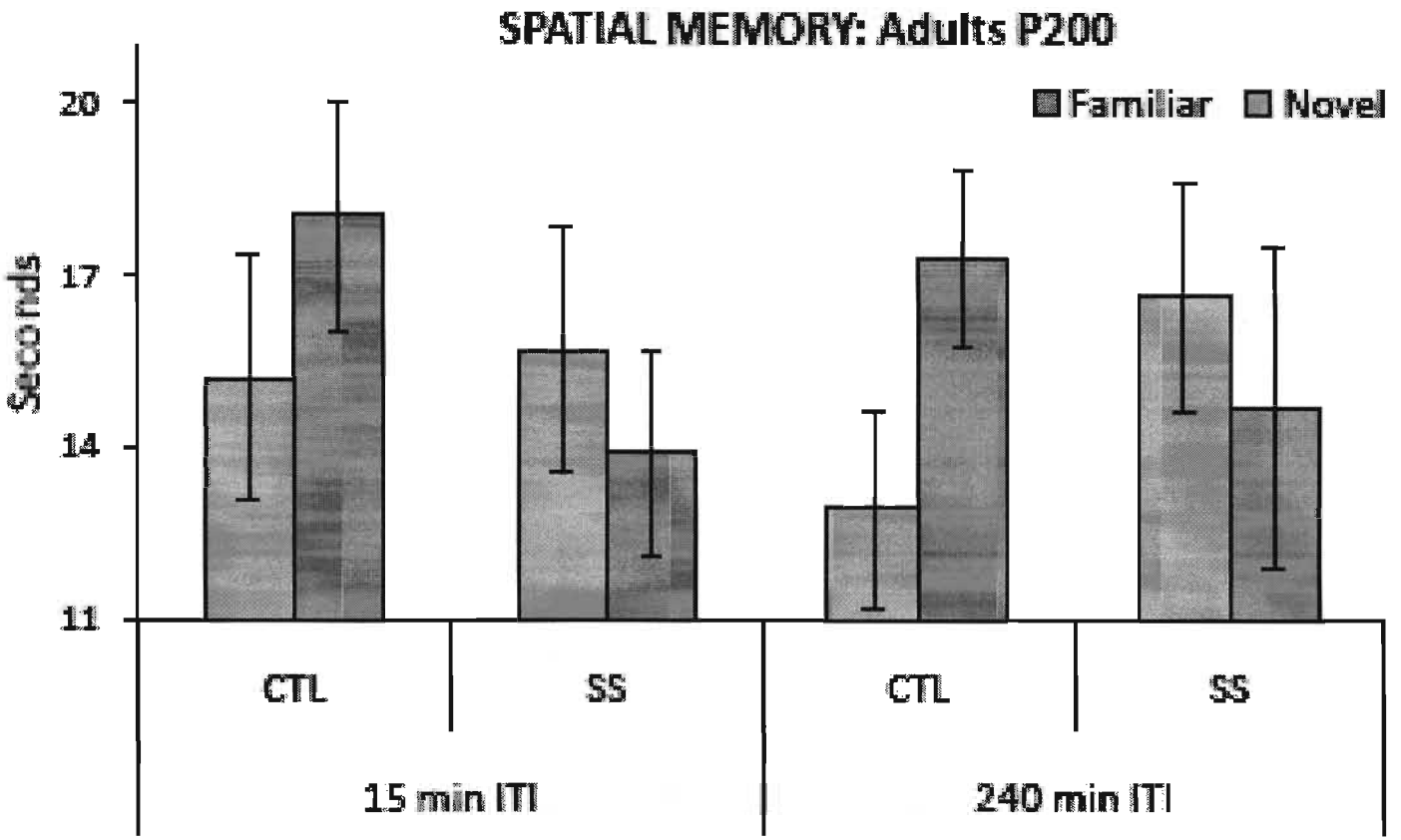


Figure 29. Experiment 3: Tested again on PND 200, spatial memory was still hindered by adolescent SS, with both 15, and 240 minute ITI.